EXPRESSION OF RETROVIRAL GENES AFTER DNA TRANSFER

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PROEFSCHRIFT

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door

Franciscus Augustinus Maria Jozef van der Hoorn
geboren te Heerlen.

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CONTENTS

Tumor virology has expanded in such a way that the deep gap between well controlled experiments on the one side and understanding of human tumorigenesis on the other side is about to be bridged. Especially the vast amount of knowledge concerning RNA tumor viruses is one of the main piers of this future bridge. The literature on these viruses has grown exponentially over the last ten years and therefore only some recent reviews will be quoted, that serve as a background for the following chapters.

RNA tumor viruses (Oncovirinae) are one of the three subfamilies of the Retroviridae and are further subdivided on the bases of their morphology as type B, type C or type D viruses (Weiss et al., 1982). Among the best studied viruses are the prototype (and almost sole representative) of the type B viruses, Mouse mammary tumor virus, and some members of the genus of type C viruses, such as the murine leukemia viruses (MLV) and the murine sarcoma viruses (MSV). The mammalian type C viruses can again be divided in a number of ways based on mode of transmission, pathogenecity, host-range and competence or defectiveness of replication. Thus MSVs are replication defective and cause solid tumors in animals as well as transformation of cells in culture. MLVs are replication competent and cause no or slowly developing neoplastic diseases of the haemopoietic or reticulo-endothelial systems.

Obviously for spread in animals or in tissue culture MSV needs a helper virus such as an MLV. There are known some 30 different transforming viruses of which only the avian sarcoma virus is replication competent (Coffin et al., 1981). They harbor a transforming gene (v-onc, v from viral). From studies in which heteroduplex mapping, nucleic acid sequencing and in vitro translation systems were employed, it follows that these viruses, mostly sarcoma viruses, are the result of rare recombination events between helper viral sequences and host cell sequences, c-onc (c from cellular). c-onc sequences have been detected in DNA of the species from which the sarcoma virus was isolated originally as well as in DNAs from related species (Bishop, 1981). c-onc sequences prove to be very much conserved during evolution. A review of the literature, indicating their possible involvement in human tumorigenesis (Rigby, 1982), is given in Chapter I.

MLV harbors three genes, one of which (pol) encodes the reverse transcriptase (RT). This enzyme formed by cleavage of an inactive precursor protein is capable of converting RNA into double stranded DNA and represents the feature that distinguishes the retroviruses from other RNA viruses. The other two genes are the gag gene and the env gene. The gag gene codes for precursor proteins, the most abundant of which is cleaved to yield the mature gag proteins p15, p12, p30 and p10, that constitute the core of the viral particle. The env gene codes for a precursor protein that upon processing yields the mature env proteins gp70 and p15(E), that make up part of the viral envelope (Stephenson, 1980). In sarcoma viruses most or all of these genes are absent.

The life cycle of the retroviruses can be roughly divided into two parts: a) the virion stage, in which the virions are built up of viral proteins and some cellular constituents and harbor the RNA genome and b) the provirus, representing the integrated state of the virus in host cell DNA. The direct precursor to the provirus is thought to be a circular double stranded DNA copy of the RNA genome that itself was formed by circularization of a linear DNA copy made by the RT after infection of cells with the virus. The provirus can be transcribed into genomic RNA and into spliced mRNAs that upon translation give rise to the precursor proteins mentioned (Stephenson, 1980). After assembly of the viral core the virions acquire their envelope through a process called budding in which the virions are shed from the cell membrane.

Although much is known about the viral proteins and their relationships to precursor proteins, some of their biological functions are still hypothetical. Also the involvement in leukemogenesis of some classes of type C RNA tumor viruses, endogenously present in the mouse genome, is still largely unknown, although many data show that one important step in this process is recombination of the exogenous retroviruses with cellular sequences, representing the env gene or parts thereof of endogenous viruses. Some of the chapters to follow give experimental details concerning biological functions coded forby the virus or the host cell and a characterization of one endogenous viral gene.

In chapter II the results are described of the study of one particular MLV, AKR virus (Akv), in rat cells. The AKR virus can only infect mouse cells and therefore belongs to the ecotropic viruses. It was introduced in

the non-susceptible rat cells by micro-injection (Graessmann et al., 1980) of Akv DNA. Its integration and expression were studied in a cloned cell line. It followed that the rat cells missed (a) function(s) necessary for faithful processing of the env precursor protein, whereas the gag precursor protein was processed normally, although relatively slowly.

The kinetics of processing of the Akv gag precursor protein was studied, as described in chapter III, in the mouse system by micro-injection of a subgenomic Akv DNA fragment, containing the gag and part of the pol gene as well as mouse cellular sequences present in the clone used. The processing appeared to be very slow compared to processing of the gag precursor proteins of other MLVs. Infection of the cells with an amphotropic MLV, that can infect mouse cells and cells from other species, indicated that the defect did not reside in the host cell. Possibly a mutation present in the Akv DNA at the junction of gag and pol genes plays a role in the defect. Analysis of cellular DNA and viral mRNA in the cells showed that a recombination had occurred between the injected DNA and endogenous MLV sequences, that are abundantly present in the mouse genome.

In chapter IV the characterization of the env gene, isolated from an endogenous MLV present in NIH-3T3 mouse cells, is described. The env gene was linked to the gag and pol genes of Moloney murine leukemia virus and transfected into mink cells. A new xenotropic MLV, XH-19, infectious to mink but not to mouse cells, resulted and was analysed. Interestingly, NIH-3T3 cells do not contain inducible MLVs and cloning of representatives of the non-inducible endogenous MLVs in NIH-3T3 cells showed that they are non-infectious in transfection experiments (Steffen et al., 1982). Our results indicate that this defect does not reside in the env gene, but in other parts of the non-inducible MLVs.

The Interlude between chapters IV and V is an homage to D.R.Hofstadter who made vast areas of mathematics and philosophy accessible to outsiders. I hope it will serve to focus the attention of the reader to some peculiar aspects of v-onc and c-onc genes.

In chapter V the molecular cloning and characterization is presented of one of the c-onc genes, c-mos(mouse), that is the cellular homologue of the transforming gene of Moloney MSV. From S1 nuclease assays and heteroduplex mapping it followed that c-mos(mouse) is almost identical to v-mos and does not contain intervening sequences.

The results reported in chapter VI were obtained by making use of the conservative nature of c-mos throughout vertebrates. The c-mos(rat) was molecularly cloned from rat DNA. Although not active by itself, it was shown to transform cells in culture after coupling the gene to promoter sequences and transfection into cells. Furthermore it appeared that not only c-mos but also sequences juxtaposed to it in rat DNA are very well conserved among eukaryotes.

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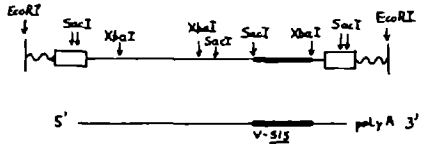
In search of human tumor genes

The material presented in this chapter is not meant to give a complete review on cellular homologues of transforming genes of retroviruses. Instead, I decided to confine myself to the most recent publications that illustrate the possible involvement of these cellular genes in the development of human tumors as well as to papers presenting molecular biological details on the relation between the transforming genes of mammalian sarcoma viruses (v-onc genes) and their cellular homologues (c-onc genes).

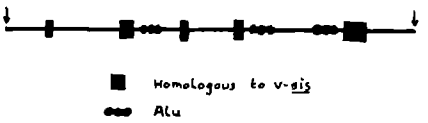
1: Mammalian v-onc and c-onc genes.

From hybridization studies in which DNA probes were used, representative for the transforming genes of sarcoma viruses, it appeared that sequences homologous to these transforming genes could be detected in many vertebrate DNAs. Not only were these cellular homologues found in DNA of the species from which the sarcoma virus under investigation was isolated originally, but also in DNA of related species (Bishop, 1981). Up till now approximately thirty different sarcoma viruses have been isolated from mammalian and avian species (Coffin et al., 1981). With the development of recombinant DNA technology it became feasible to isolate DNA clones of these transforming viruses. Recently a number of c-onc genes have been cloned from DNA of several vertebrates including man. Some of these c-onc genes that are described in more detail here have been implicated in human tumorigenesis or were analysed in great detail. Others such as c-abl, the cellular homologue of the transforming gene of Abelson murine leukemia virus, will not be described because no or very few data were published.

a: v-sis/c-sis. Simian sarcoma virus (SSV) and its associated helper virus (SSAV) were isolated from a fibrosarcoma of a wooly monkey by Theilen et al. (1971). SSV is tumorigenic in primates and induces transformation of cells in culture. SSV DNA was molecularly cloned from circular viral DNA (Gelmann et al., 1981) and as integrated proviral DNA (Robbins et al., 1981). The SSV specific sequences (0.9 kbp in size) could be positioned at the 3'end of the SSV genome. No sis specific protein has been detected yet.



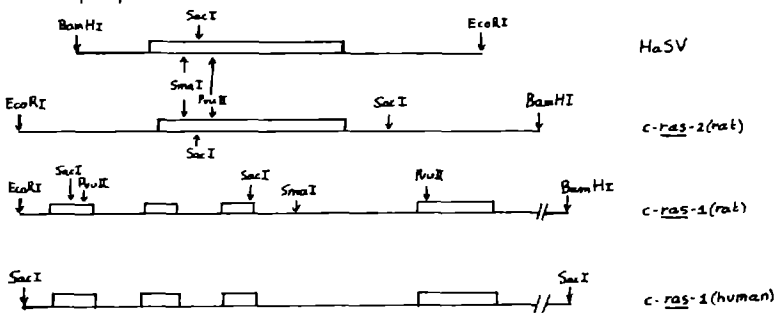
Organization of integrated SSV DNA. After Robbins et al.,1981.



Organization of c-sis(human) DNA. After Dalla-Favera et al.,1981.

The recombination events that led to the appearance of SSV took place between cellular sequences from the wooly monkey and the env region of SSAV. Using SSV DNA as a probe Dalla-Favera et al.(1981) identified and cloned the human c-sis gene. In contrast to the viral sis gene, the human c-sis gene extended over 12 kbp and contained four intervening sequences. Interestingly, the authors report the observation of the presence of repeated sequences in the c-sis introns that are related to the Alu family of repeats. The members of this Alu family are estimated to be present approximately 300,000 times throughout the human genome (Jelinek et al., 1980). As will be described later, investigators make use of the Alu DNA as probe to look for human onc genes in DNA transfer experiments. Recently the sequence of v-sis was determined by Devare et al.(1982). They found a 675 nucleotide-long open reading frame and deduced the amino acid sequence of the putative sis protein.

b: v-ras/c-ras. Harvey murine sarcoma virus (HaSV) was isolated by Harvey (1964) from a rat, injected with Moloney murine leukemia virus and was molecularly cloned by Hager et al.(1979), who used circular viral DNA for this purpose.

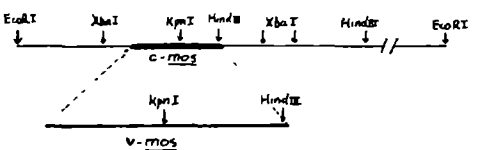


Schematic restriction maps of HaSV, c-ras-1(rat) and c-ras-2(rat) (after DeFeo et al., 1981) and of c-ras-1(human) (after Chang et al., 1982).

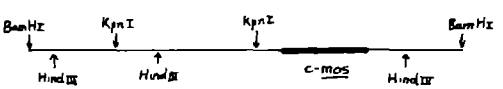
By using heteroduplex mapping and DNA transfer experiments it was determined that the HaSV transforming sequences were located at the 5'end of the HaSV genome. Antisera obtained from tumor bearing rats injected with HaSV, were used to identify the v-ras gene product, a 21,000 D protein with phosphokinase activity (Shih et al., 1979). Recently monoclonal antibodies were developed (Furth et al., 1982), that recognize determinants

on p21 or p23 (a p21 processing product) specific for HaSV and for Kirsten murine sarcoma virus (KiSV, closely related to HaSV) or on $p21^{bas}$, the product of the transforming gene of BALB MSV, v-bas, a gene also closely related to v-ras (Andersen et al., 1981). Using a clone containing only v-ras sequences DeFeo et al.(1981) isolated two rat cellular homologues of v-ras. Of these c-ras-1(rat) was found to contain intervening sequences, while c-ras-2(rat) did not contain any intervening sequences. The same DNA probes were used to isolate four human homologues of v-ras (Chang et al.,1982). One of these, c-ras-1(human), showed a large similarity in organization to c-ras-1(rat). Both these c-onc genes appeared to code for a 21 kD protein, that could be identified as very closely related to the transforming protein of HaSV.

c: v-mos/c-mos. Moloney murine sarcoma virus (M-MSV) was isolated from a Balb/c mouse injected with Moloney murine leukemia virus (Moloney, 1966). M-MSV induces cellular transformation in cultured fibroblasts and fibrosarcomas in animals. M-MSV DNA was cloned by Verma et al.(1980). The location of the transforming gene (v-mos) had been determined before by heteroduplex mapping (Hu et al., 1977) and DNA transfer assays (Andersson et al., 1979). Using v-mos specific DNA probes Jones et al.(1980) and Oskarsson et al.(1980) isolated the mouse cellular homologue c-mos(mouse). In contrast to most other c-onc genes, c-mos(mouse) was found to consist of one uninterrupted stretch of 1159 nucleotides, coding for a 374 amino acid protein (Van Beveren et al., 1981). An antiserum raised against a synthetic C-terminal peptide, was used to identify the v-mos gene product in M-MSV transformed cells (Papkoff et al., 1982). Recently Watson et al. (1982), using a v-mos specific DNA probe, isolated the human homologue, c-mos(human). It appeared to be 77 % homologous to c-mos(mouse) and to code for a 346 amino acid protein. Like c-mos(mouse) c-mos(human) did not contain intervening sequences.

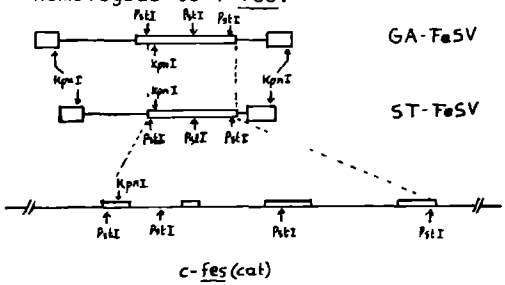


Schematic restriction map of v-mos and c-mos(mouse) (after Jones et al. 1980).

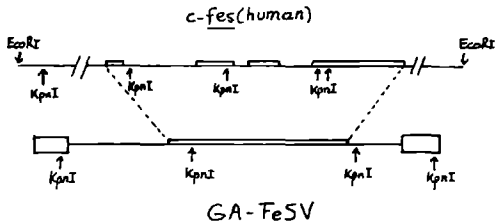


Restriction map of c-mos(human) (after Watson et al.,1982).

d: v-fes/c-fes. Two independently isolated, but very similar strains of Feline sarcoma virus were described by Snyder & Theilen (1969, ST-FeSV) and Gardner et al.(1970, GA-FeSV). The viruses produce fibrosarcomas in cats and transform cells in culture. ST-FeSV encodes a 85 kD polyprotein (Ruscetti et al., 1980) which shows protein kinase activity (Van de Ven et al.,1980) and is encoded in part by the v-fes sequences (Sherr et al., 1979). ST-FeSV was molecularly cloned by Sherr et al.(1980). The transforming sequences appeared to be 1.3 kbp long and were used as probe by Franchini et al.(1981) to isolate the cat cellular homologue. c-fes(cat) appeared to consist of four exons and three introns, the exons being homologous to v-fes.



Restriction maps of ST-FeSV and c-fes(cat) (after Franchini et al., 1981) and GA-FeSV (after Fedele et al.,1981).



Restriction map of c-fes(human) (after Groffen et al.,1982).

The integrated proviral GA-FeSV DNA was cloned by Fedele et al.(1981). By heteroduplex mapping and restriction enzyme analysis they defined the position of the v-fes sequences that are common to both ST- and GA-FeSV. v-fes of GA-FeSV appeared to contain an additional 450 bp DNA 5´to the v-fes sequences of ST-FeSV. Using the same v-fes specific DNA probes Groffen et al.(1982) isolated the human c-fes sequences from a human cosmid library. c-fes(human) showed an organization very similar to c-fes(cat): it also contained exons and introns, the size of the two genes being approximately the same.

2: DNA transfer techniques.

In short two principle methods for the introduction of DNA into cells in culture will be described, because these techniques have proved to be essential for the determination of the biological activity, such as transforming activity, of the DNA under investigation. More experimental details and possibilities have been recently described by Graessmann et al.(1980) and Van der Eb & Graham (1980).

a. transfection. The method described here was first published by Graham & Van der Eb (1973). In short: DNA under investigation is dissolved in a phosphate buffer together with carrier DNA. The pH of the buffer is extremely critical. Addition of $CaCl_2$ results in the appearance of a microscopic precipitate that renders the solution opalescent. The precipitate, containing co-precipitated DNA, is applied to cells incubated with medium for several hours and removed again. The cells can then be passaged or treated with DMSO (Stow & Wilkie,1976) which in some unknown way enhances the transfection efficiency.

The transfection efficiency is such that only a very small number of cells (approximately 1 in 10,000) ultimately integrate and express the exogenous DNA. Compatibility to transfection seems to be a function of the physiological state of the cell. The exact mechanisms taking place during the procedure are not yet completely understood. Loyter et al.(1982) using DNA labeled with fluorescent dyes, showed that in fact most cells take up the applied DNA in their cytoplasm, but only 1 % of these have any detectable exogenous DNA in their nucleus. Further they showed that calcium phosphate increased the DNA concentration on the cell surface and protected DNA against nucleases in the serum.

There is evidence that the exogenous DNA first integrates into the carrier DNA used, forming high molecular weight structures, before integrating into the host cell DNA (Perucho et al.,1980). Also frequently the ends of the exogenous DNA are found to have undergone deletions after transfection and recombination events were detected occurring between DNAs in co-transfection experiments (Anderson et al.,1982). Transfection can only be used in the following cases. 1) the transfected DNA contains a marker gene that can be selected for after transfection, such as a thymidine kinase gene or a transforming gene or 2) expression of the DNA after transfection results in production of virus that can spread through the culture.

b. micro-injection. Graessmann (reviewed by Graessmann et al.,1980) and Diacumakos (1973) described the use of glass microcapillaries for the introduction of DNA into single cells. In principle capillaries are drawn on a needle puller so that they acquire tips of less than 0.5 µm. They are mounted on the holder of a Leitz micromanipulator and filled with a DNA solution either from the back (Diacumakos) or by a suction device (Graessmann) Cells to be injected are plated on glass slides or petridishes and are injected under a phase contrast microscope. Injections into

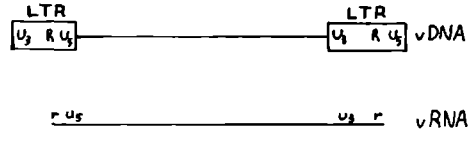
the cytoplasm can be followed by movement of cytoplasmic particles and injections into the nucleus are visible by a clear shift in refractive index. The estimated injection volume is 10^{-11} ml, which is 5-10 % of the cell volume. After injection of 1-10 molecules of retroviral DNA in the nucleus 0.1-10 % of the cells expressed the DNA, whereas practically every cell expressed the DNA after nuclear injection of 100 molecules per cell (Kopchik et al.,1981). These authors also showed that injected DNA is transcribed as early as four hours after injection.

Although micro-injection clearly can only be performed on a relatively small scale, the high efficiency of expression allows the use of DNAs that do not contain a selectable marker. A relatively small amount of cell cloning and screening of the injected cells leads to the establishment of injected cell lines.

The micro-injection technique was also used to introduce DNA into isolated mouse eggs that were then transplanted back into foster mothers and resulted in mice harboring in their chromosomal DNA new exogenous genes. Thus the human β-globin gene and a viral thymidine kinase gene were introduced in mouse eggs by Wagner et al.(1981), a fusion gene consisting of the metallothionein promoter and a viral thymidine kinase gene was introduced by Brinster et al.(1981) and the human insuline gene was introduced by Bürki & Ullrich (1982). The first two papers report the expression of the introduced DNA in offspring mice, while all reports show detection of only 1-50 integrated copies of the DNA used, although up to 30,000 molecules were injected per mouse egg.

3: Eukaryotic functions in retroviral long terminal repeats. Activation of genes.

The coding region of integrated proviruses is flanked on either side by identical viral sequences, long terminal repeats (LTR), that arose as a consequence of the replication mechanism of retroviruses (Temin,1982). The LTR contains unique sequences originating from the 3´end of the viral RNA and from the 5´end of the viral RNA. In between is a short stretch of nucleotides, called R, that is present on both sides of the viral RNA. The LTRs from a large number of retroviruses have been sequenced and although the sequences are different for different retroviruses, they do have some features in common that shed light on the function of the LTR. Some of the features important for understanding



of the LTR as an activating sequence, will be mentioned here briefly.

a) By comparing the LTR sequences with sequences that are assumed to represent signals involved in eukaryotic transcription, some functions could be assigned to parts of the LTR. Thus signals for initiation of transcription (Hogness box, Gannon et al.,1979) and signals for polyadenylation and termination of transcription (Konkel et al.,1978) were found. HeLa cell-free extracts offered the possibility to examine the proposed functions (Manley et al.,1980). Indeed Ostrowski et al.(1981) using HaSV DNA and Fuhrman et al.(1981) using M-MLV DNA detected the expected in vitro RNA species after incubation of these DNAs in the HeLa cell-free extract. Apparently, the in vitro used RNA polymerase II initiation sites are identical to the ones operative in vivo.

b) From the sequence data it appeared that in some cases a small part of the 3'unique sequences of the LTR is present as a direct repeat. The sizes and locations of the repeats vary (Dhar et al.,1980, Van Beveren et al.,1982). A similar repeat was reported to be present in the promoter region of SV40, a DNA tumor virus belonging to the papova viruses. It seemed to play a role in initiation of early gene transcription in vivo (Benoist & Chambon,1981). Gruss et al.(1981) , using deletion mapping in this SV40 promoter region, showed that SV40 DNA having only one repeat was still viable upon transfection, but viability was lost if deletions were present in this one repeat. The role of the 72 bp repeat present in the LTR of M-MSV (Dhar et al.,1980) was investigated by replacing the SV40 72 bp repeat by the M-MSV repeat. Levinson et al. (1982) showed that after this replacement SV40 DNA is still completely viable, although there was no sequence homology between the two repeats at all. Apparently they fulfill the same function and they are named enhancer sequences.

c) With the exception of the Mouse mammary tumor virus (MMTV) LTR, none of the LTRs sequences thus far contain an open reading frame, although the 3'unique region can be quite large. The MMTV LTR harbors in the large U_3 region an open reading frame of 960 nucleotides, that could code for a 36 kD protein. This protein has not yet been found in MMTV infected cells (Fasel et al.,1982, Kennedy et al.,1982). In an earlier report Dickson et al.(1981) showed that MMTV DNA can direct in vitro synthesis of RNA and proteins, among which was a 36 kD protein, most probably coded for by the U_3 sequences. Furthermore, it appeared that MMTV gene expression

was glucocorticoid dependent, both in mammary tumors (Parks et al.,1974) and in cells transfected with MMTV DNA (Buetti & Diggelmann,1981). Fasel et al.(1982) showed that the hormone sensitivity is located in the MMTV LTR. Other examples of glucocorticoid sensitive gene expression were found for the human growth hormone gene (Robins et al.,1982) and the rat metallothionein gene (Mayo & Palmiter,1981).

The putative function of the eukaryotic signals found in the LTR was assayed by combining the LTR sequences with genes, whose expression could be selected for and that either were deleted in their own promoter region or were inactive by themselves in transfection experiments. The transforming genes of retroviruses and their cellular homologues were the first to be used for this purpose (Oskarsson et al.,1980, Blair et al.,1981, DeFeo et al.,1981). Another, widely used, gene that can be selected for is the thymidine kinase gene (TK) of Herpes simplex virus-1 that was cloned by Enquist et al.(1979) and sequenced by Wagner et al.(1981). TK^- cells can be transformed with TK DNA to the TK^+ phenotype (Wigler et al.,1978). Selection media containing aminopterin select for the TK^+ cells (Summers et al.,1975). Thus Joyner et al.(1982) showed that the LTR of Spleen focus forming virus could successfully replace part of the promoter region, including the Hogness box, of the TK gene. The LTR proved to be functional only if oriented in the same direction as the coding strand of the TK gene.

The same type of experiments was used to study the expression of genes under hormonal control, by combining the gene under investigation with the MMTV LTR. It appeared that after co-transfection of the MMTV LTR with the TK gene and selection for TK^+ cells, the transcription of the MMTV open reading frame was hormone sensitive (Fasel et al.,1982). Also the mouse dihydrofolate reductase cDNA clone could be expressed in a hormone dependent way by linking it to the MMTV LTR (Lee et al.,1981).

Therefore LTRs contain functional eukaryotic signals, necessary for faithful transcription of linked genes. Also, in some cases, they contain direct repeats in the U_3 region that can act as enhancer sequences. In a comparison with the strong Adenoviral 2 late promoter and weak promoters present in the ovalbumin gene (Wasylyk et al.,1980) and the mouse globin gene (Luse & Roeder,1980) the LTR promoter is classified as weak.

4: Activation of c-onc genes.

As referred to in the previous section v-onc and c-onc genes were the first to be used in LTR activation experiments. Thus Blair et al.(1980) showed that v-mos can be activated when coupled to the M-MSV LTR. The LTR could be positioned 5´ or 3´with respect to v-mos and could be either ligated or co-transfected with v-mos. v-mos could be activated when isolated from cloned M-MSV DNA or from cloned cDNA (Donoghue,1982). From cloning of c-onc genes it appeared that they are not transforming themselves when transfected into NIH-3T3 cells. LTRs can be used to activate these c-onc genes. Blair et al.(1981) showed that c-mos(mouse) was transforming when ligated to LTR sequences, that could be positioned as far as 1500 bp upstream from c-mos(mouse). Using a similar experimental design DeFeo et al. (1981) used an LTR to activate the c-ras-1(rat) gene that is built up of four exons and three introns. The transfection efficiency was low when compared to the efficiency for the v-ras gene; in the transformed cells p21, the v-ras gene product, was clearly detectable. Chang et al.(1982) observed the expression of a closely related p21 protein in cells transfected with DNA consisting of an LTR and c-ras-1(human), that also contains three intervening sequences. Furthermore, it appeared that a very slight increase in the amount of p21 in cells could apparently cause transformation. Thus NIH-3T3 cells transformed with LTR-c-ras-1(human) DNA contained only four times as much p21 as did normal NIH-3T3 cells. However, using monoclonal antisera, that discriminate between the closely related KiSV p21 or HaSV p21 proteins, Furth el al.(1982) and Chang et al.(1982) found that the p21 observed in normal rodent cells is related to the KiSV p21, while the normal human cells diplay expression of HaSV related p21 protein.

Recently two publications reported that two cloned human c-onc genes could not be activated with promoter sequences. Groffen et al.(1982) showed that although most or all of the v-fes related sequences were present in clones of human c-fes, it proved to be imossible to activate this gene. For activation experiments they used an SV40 promoter/TK selection system, that was previously shown to be functional in an assay using the globin gene. However, they could not yet exclude the possibility of very small mutations in the c-fes(human) that would abolish the transforming activity. Watson et al.(1982) isolated the c-mos(human) gene and detcted the presence of an open reading frame. However, they failed to

transform NIH-3T3 cells with c-mos(human) coupled to an LTR. Also the hybrid molecule consisting of an LTR, the 5'end of c-mos(mouse) and the 3' end of c-mos(human) failed to transform cells after transfection. In the case of c-mos(human) this failure is not due to mutations. The very conservative character of the c-onc genes, such as c-mos and c-fes, implies that they fulfill some, yet unknown, important function(s) in cell growth regulation or cell differentiation. If the failure to transform cells with the DNAs mentioned in the last two papers, is not due to mutations, this might indicate that the proposed regulatory function(s) is different from the transforming function. Apparently at least in some cases the transforming function might be lost. In other cases, for instance c-ras-1 expression, transformation seems to result from increases in the expression of the c-onc gene.

5: c-onc genes involved in human tumorigenesis.

The observation that c-onc genes, activated by coupling to eukaryotic promoter sequences, can cause cell transformation in vitro, suggested that they could play a role in non-viral tumorigenesis. Several methods based on transfection were developed for the identification of such genes. In short: DNA, isolated from human tumors or from cell lines derived from tumors, is transfected onto NIH-3T3 cells. DNA, isolated from primary foci, is again transfected onto NIH-3T3 cells. By making use of the ubiquitous character of the Alu repetitive sequences (Jelinek et al.,1980), that are absent from NIH-3T3 DNA, it is possible to specifically detect human DNA in the secondary NIH-3T3 foci. For a more detailed characterization of the human c-onc gene DNA from the tumor cell lines, the primary and the secondary foci are analysed by restriction enzyme analysis. Some enzymes will leave the transforming activity of the DNA intact, while others will destroy it. Indeed this approach led to the discovery of transforming sequences in some cell lines derived from human tumors.

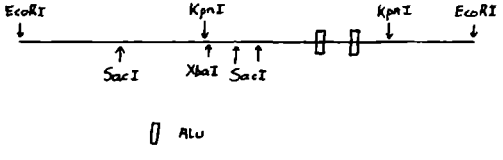
Krontiris & Cooper (1981) examined a large number of human tumors and tumor cell lines for the presence of transforming sequences and found this activity for the bladder carcinoma cell lines EJ andJ82. The transforming activity was destroyed in both cases with EcoRI, HindIII or KpnI. Using a similar experimental design Murray et al. (1981) demonstrated that the transforming genes present in colon, bladder carcinoma and promyelocytic leukemia cell lines were different ones. In both reports a probe, detecting the Alu sequences, was used for characterization of the transforming genes. Based on the inactivation of transforming activity by certain

restriction enzymes, Lane et al. (1981) observed the same or closely related transforming sequences to be present in a number of MMTV induced mouse mammary tumors, a carcinogen induced mouse mammary tumor and a cell line derived from a human breast cancer, MCF-7. Recently Becker et al. (1982) detected the presence of a 82 kD glycoprotein that is specifically associated with the transmissible transforming gene of the MCF-7 cell line. Similarly Perucho et al. (1981) observed the expression of a common transforming gene in cell lines derived from two lung carcinomas and one colon carcinoma; this gene appeared to be different from the transforming genes present in a bladder carcinoma cell line, T24, and a neuroblastoma cell line. Unfortunately, from all data presented in the papers mentioned above it appeared that up till now none of the tumor DNAs tested contained transforming sequences while only a small number of tumor derived cell lines harbored detectable transforming genes.

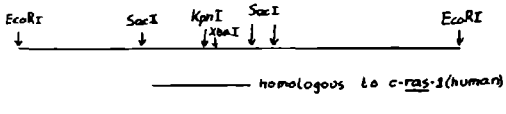
The availability of a large number of different probes, isolated from DNA clones of sarcoma viruses, allowed the analysis of RNA species in different tumor cell lines. Eva et al. (1982) detected a sis related RNA species of 4.2 kb in glioblastoma and sarcoma cell lines, but not in cell lines derived from melanomas, carcinomas and teratomas. Surprisingly, all cell lines investigated, among which were normal human cell lines, showed expression of c-myc, c-abl and c-bas related RNA species to a different, but clearly detectable, degree (c-myc is the cellular homologue of the transforming gene of MC-29, an avian acute leukemia virus (Coffin et al.,1981)). Westin et al. (1982[a]) found RNA transcripts homologous to abl, ras and myc sequences in all fresh leukemias and derived cell lines investigated. They observed a variation in expression of only two to three fold with the exception of the HL60 promyelocytic leukemia cell line, that showed a 10 fold increase in expression of myc related RNA. The probability of a role for c-myc in the differentiation of haemopoietic cells was demonstrated by the suppression of c-myc expression in DMSO treated HL60 cells. HL60 cells differentiate to granulocytes when treated with DMSO (Collins et al., 1979) or to macrophages when treated with phorbol ester (Rovera et al., 1979). A similar role for c-amv, the cellular homologue of the transforming gene of Avian myeloblastosis virus (Duesberg, 1979), in the differentiation of haemopoietic cells was suggested by Westin et al.(1982[b]). They investigated

the expression of c-amv in a large number of neoplastic cell lines, representing different stages of B- and T- lymphocyte differentiation. Lane et al.(1982), investigating pre B-, mature B-, intermediate T- and mature T-tumor cell lines, demonstrated the existence of transforming genes specific for each of the differentiation stages, based on restriction enzyme inactivation experiments as well as on the presence or absence of repetitive sequences closely associated with the transforming genes. However, although c-amv expression is specific for certain differentiation stages of haemopoietic cells it is not necessarily the cause of the transformed state of the cells, because Lane et al. (1982) did not detect transforming activity in the DNA of a tumor cell line, MOLT-4, in which Westin et al. (1982) observed a very high expression of c-amv.

Recently several groups reported the isolation of the transforming gene from the bladder carcinoma cell lines T24 and EJ. Goldfarb et al. (1982) used the gene rescue method, with the Ecoli tRNA amber suppressor gene supF as a marker, to isolate the transforming gene from the cell line T24. They detected a restriction enzyme polymorphism of the region close to the T24 onc gene among different human DNAs, but no major rearrangements were found in the T24 onc gene when compared to human placental DNA. Shih & Weinberg (1982), using the Alu sequences as probe, isolated the EJ onc gene, that proved to be very similar to the T24 onc gene. Similar results were reported by Pulciani et al.(1982) for the T24 onc gene. Parada et al. (1982), Der et al. (1982) and Santos et al. (1982) found the T24 and EJ onc genes to be homologous to v-ras. When comparing their data with data from Chang et al. (1982) who reported the isolation of c-ras-1(human) it appears that the T24/EJ onc gene are identical to c-ras-1(human). Santos et al. (1982), however, found a 200 bp deletion outside but close to the 3' end of the T24 onc gene when compared to c-ras-1(human).



Organization of the EJ onc gene (after Shih & Weinberg, 1982)



Organization of the T24 onc gene (after Parada et al.,1982)

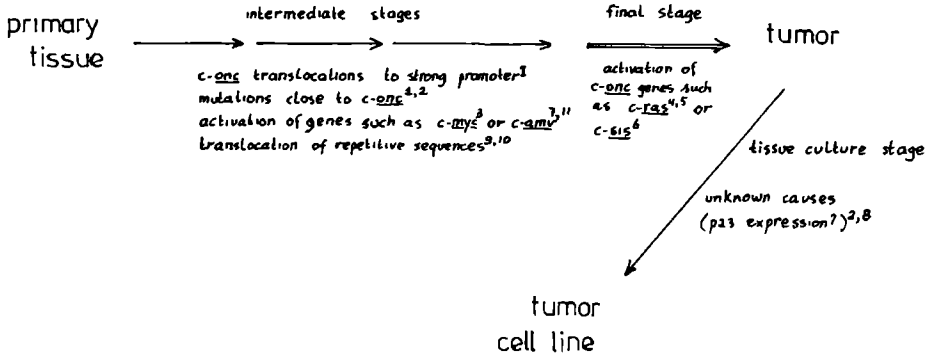
The mechanism by which the c-ras-1(human) is activated, thus possibly causing transformation of bladder cells, is not clear. Mutations, not detectable by restriction mapping, could activate the gene thus causing a slight increase in its expression that might suffice for transformation (Der et al., 1982). Alternatively transformation might be the result of qualitative changes instead of quantitative changes of p21. Interestingly, the phosphoprotein p23, a processing product of p21 (Furth et al., 1982), is absent from most human tumor cell lines. It is present, however, in T24 cells and in NIH-3T3 cells transformed by the T24 onc gene (Santos et al., 1982). This suggests that the appearance of p23, more than the presence of p21, is related to the transformed state of the bladder carcinoma cells. Also it is unclear whether or not the Alu repetitive sequences present at 2.0 kbp from the c-ras-1(human) gene that appeared to be mobile in cloning experiments, play a role in transformation (Shih & Weinberg, 1982). A close association of Alu sequences and c-sis(human) was reported previously by Dalla-Favera et al. (1981).

6: Concluding remarks.

Some remarks should be made concerning the possibility of artifacts due to the experimental approach chosen. With these remarks in mind it is possible to draw a very schematic figure showing the data presented.

First of all, the isolation of the c-onc genes is dependent upon a transfection assay in which visible foci can be picked up. For this purpose NIH-3T3 cells are used by all groups. However, up till now no foci resulted from transfection of tumor DNAs and only a small number of tumor derived cell lines contained transforming sequences. Furthermore, it appeared that the c-onc genes isolated thus far from tumor cell lines could transform Rat-1 cells and CHEF/18 cells (Smith et al., 1982) in addition to NIH-3T3 cells. NIH-3T3 cells are almost transformed themselves, requiring only one event for complete transformation (Rigby, 1982, Newmark, 1982), but the other two cell lines are more normal in that multiple events are required for complete transformation. Therefore, the activation of the c-onc genes isolated up till now, most probably represents the final step in the multiple stage tumorigenesis process.

Secondly, at least in two cases it appeared that the c-onc that is thought to be the cause of the particular tumor development investigated, is different from the c-onc gene that resulted from the transfection assays used. Thus activation of c-myc was shown to cause bursal lymphomas in



1)Klein, 1981
2)Santos et al., 1982
3)Hayward et al., 1981
4)Smith et al., 1982
5)Chang et al., 1982
6)Eva et al., 1982
7)Westin et al., 1982[b]
8)Furth et al., 1982
9)Humphries, 1981
10)Shih & Weinberg, 1982
11)Baluda & Goetz, 1961

chickens (Hayward et al., 1981), but a gene very similar to c-ras-1(human) was isolated in transfection assays with the lymphoma cell line DNA (Cooper & Neiman, 1981, Rigby,1982). Secondly, although c-amv was shown to be highly expressed in a human tumor cell line, MOLT-4, no c-onc gene could be isolated in the transfection assays (Westin et al., 1982[b], Lane et al.,1982).

Thridly, it is not yet known what differences exist between tumor cells and cells from tumor derived cell lines. In general, it proves to be very difficult to establish a permanent cell line from tumors. This could imply that subtle differences in growth properties or substrate requirements by activation of genes take place in this process or are selected for from a heterogenous population of primary tumor cells. Possibly some of these genes are isolated in the transfection experiments. Small differences such as the 200 bp deletion in the vicinity of c-ras-1(human) or rearrangement of Alu sequences next to c-ras-1(human) have been reported (Santos et al., 1982, Shih & Weinberg, 1982).

Finally, small elevations of expression of c-onc genes were implied in tumorigenesis. Der et al.(1982) showed that a two to four fold increase in c-onc expression resulted in transformation of cells in the case of c-ras.

A similar barely detectable expression of c-mos or v-mos also resulted in transformation of cells (Papkoff et al., 1982). But the finding that slightly modified c-onc products, such as p23(ras), are found in some human tumors, instead of the normal endogenous p21(ras), indicates that not only quantitative but also qualitative differences will be found to play a role in the development of tumors (Klein, 1981).

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CHAPTER II

3Y1 Rat Cells are defective in Processing of the Envelope Precursor Protein of AKR Virus

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ABSTRACT

Rat 3Y1 cells were infected by AKR virus through micro-injection of molecularly cloned proviral DNA. Based on a strong immunofluorescence using anti-p30 as an antiserum a cell clone (RESA-2) was selected that had a high expression of viral antigens. Subsequent restriction analysis of its DNA revealed that the RESA-2 clone contained at least 30 apparently intact integrated proviruses per genome. There was an apparently normal synthesis and processing of gag- and pol-gene products. The viral envelope precursor polyprotein $gPr82^{env}$, however, did not yield the major envelope glycoprotein gp70. The gag-precursor polyprotein, $Pr65^{gag}$, as well as the $gPr82^{env}$ from RESA-2 cells were identified as AKR viral proteins by gelelectrophoresis of hydroxylamine cleavage fragments. The virions formed by RESA-2 cells lacked gp70 and were non-infectious. After fusion of RESA-2 cells and mouse cells an infectious N-tropic virus was produced. The results indicate that rat 3Y1 cells lack (a) factor(s) necessary for the correct processing of $gPr82^{env}$. The high incidence of abortive infections of murine leukemia virus (MLV) in susceptible rat cells reported by others is therefore probably due to defective particles in the virus stock and/or to the lack of (a) cellular factor(s) necessary for reverse transcription and subsequent integration of the viral genome.

Compared to the number of reports dealing with mouse retroviruses in the mouse system, very few publications report experiments in which mouse retroviruses are studied in rat cells. Rat cells have the advantage that no endogenous viruses are present that are homologous to the well characterized ecotropic viruses such as Moloney murine leukemia virus (M-MLV) (1) or Friend murine leukemia virus (F-MLV) (2). Not all rat cell lines are susceptible to infection with these viruses: NRK cells,however,

can be infected and were used in the studies mentioned (3). The data from these reports show that although very high multiplications of infection were used only one provirus (in the case of non-producer clones) or a low number of proviruses (in the case of producer clones) were present (1, 4). Furthermore, the percentage of clones showing abberant expression of the retroviruses was high (5). This observation, however, does not necessarily mean that the rat cells and the murine viruses are less compatible since similar defects were found when single mouse cells were infected (3).

We set out to establish a rat cell line containing many Akv proviruses in order to distinguish between possible early and late defects occurring during infection of rat cells with mouse ecotropic viruses. For this we made use of micro-injection (6) of molecularly cloned proviral Akv DNA (7) into a non-susceptible rat cell line, 3Y1.

3Y1 cells could not be productively infected with AKR virus, as was concluded from XC- and reverse transcriptase (RT) assays and from the lack of viral protein synthesis in 3Y1 cells exposed to AKR virus (data not shown). Therefore, after succesful micro-injection of 3Y1 cells with Akv DNA any integration of Akv proviruses would be the result of the micro-injection and not of secondary infections. The Akv DNA clone used (7), clone 623, contained a complete, infectious, copy of Akv as well as mouse cellular sequences. From the injection volume (approximately 10^{-11} ml) and the DNA concentration used (0.1 mg/ml) we estimate that 50 molecules are injected per cell. We used an indirect immunofluorescence assay to detect expression of p30, the major viral core protein, in injected cells , as described (8). After injection of 100 cells in the nucleus with Akv DNA we found approximately 5-10 % of the cells to be immunofluorescence positive and through single cell cloning we obtained a cell line, named RESA-2, that appeared to be very positive for expression of p30.

DNA was isolated from this cell line and from 3Y1 cells and analysed by restriction analysis for the presence of Akv DNA. From Fig.1 it can be seen that 3Y1 cells do not contain sequences homologous to the Akv sequences (lanes A, B and C). From lane D the number of proviral Akv

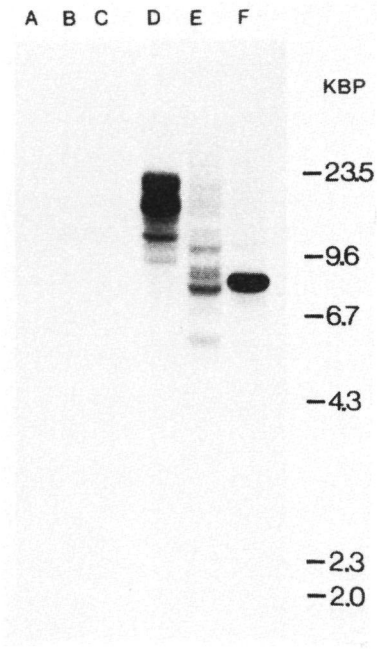

Figure 1:Analysis of Akv DNA in RESA-2 cells. High molecular weight DNA was isolated as described (9) from 3Y1 cells (lanes A, B and C) and from RESA-2 cells (lanes D, E and F). The DNAs were digested with EcoRI (lanes A and D), XhoI (lanes B and E) or with PstI (lanes C and F) and separated on a 0.7 % agarose gel (SeaKem). After transfer to a nitrocellulose filter (Schleicher & Schüll, BA 85) according to Southern (10) Akv specific DNA was detected by hybridization in 50 % formamide with nick translated clone 623 DNA as described (8). Exposure of blots was on Kodak XR-5 film for 3 days at -70°. HindIII digested λ DNA was used as a marker.

copies can be estimated, because EcoRI does not cleave Akv DNA. From this Fig. and from autoradiographs of similar blots of low percentage gels we estimated that 30 Akv copies are present in RESA-2 cells. Except for one, all bands are of a size greater than genome size (8.8 kbp). XhoI cleaves Akv DNA in the middle and thus generates many bands, some smaller than genome size (lane E). To show that most copies contain genome size Akv DNA we digested RESA-2 DNA with PstI, that only has cleavage sites in the long terminal repeats (LTR) of Akv DNA, thus generating a band of 8.2 kbp if there are no major deletions in the Akv DNA. Lane F shows that almost all copies of the Akv DNA generate a band of 8.2 kbp. Only three smaller bands are seen. Therefore in RESA-2 cells approximately 30 apparently complete Akv DNA copies are present.

The use of the immunofluorescence assay in the cloning of the cell line RESA-2 implied expression of Akv gag related proteins. This expression was investigated in more detail by pulse-chase experiments. The results are shown in Fig.2. The lanes with the antisera used, were grouped in the same order in which the mature viral proteins are encoded on the Akv genome,

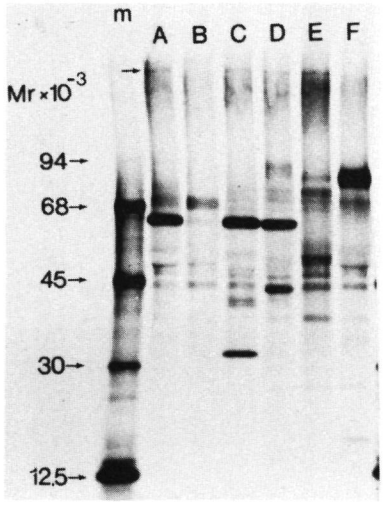

Figure 2: Viral protein expression in RESA-2 cells. RESA-2 cells were labeled with (^{35}S)-methionine (75 µCi/ml) for 30 min and chased for 3 hr. After lysis in PBSTDS, according to Van Zaane et al.(11), viral proteins were immunoprecipitated by incubation of portions of the lysate first with the antisera anti-p15 (lane A), anti-p12 (lane B), anti-p30 (lane C), anti-p10 (lane D), anti-RT (lane E) or anti-p15(E)p12(E) (lane F) and next with protein A-Sepharose (Pharmacia). The precipitates were analysed by electrophoresis on a 7-18 % SDS polyacrylamide gel. m denotes the marker lane.

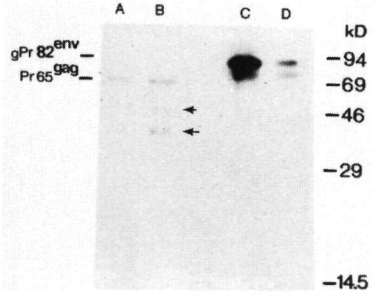

Figure 3: Identification of viral precursor proteins. In order to compare the gag- and env-precursor proteins in RESA-2 cells and in NIH-3T3 cells productively infected with AKR virus, slices were cut out from gels on which the precursor proteins present in these two cell lines had been separated. The slices were treated with hydroxylamine (Saris et al., manuscript in preparation), loaded on a 10 % SDS polyacrylamide gel on which the cleavage fragments were separated by electrophoresis. Lane A, Pr65^{gag} from RESA-2 cells. Lane B, Pr65^{gag} from NIH-3T3(Akv) cells. Lane C, gPr82^{env} from RESA-2 cells. Lane D, gPr82^{env} from NIH-3T3(Akv) cells.

i.e. p15, p12, p30, p10, RT, (gp70-p15(E)). The anti-p15, anti-p12, anti-p30 and anti-p10 antisera detect the gag-precursor protein Pr65^{gag}. The fainter band in lane B is due to the low titer of the anti-p12 antiserum. In lane C p30 can be seen. Bands representing p15 and p10 are not detected because these proteins do not contain methionine. In lane D the intermediate cleavage product Pr40^{gag} can be seen. In lane E the Pr180$^{gag\text{-}pol}$ precursor

protein is visible after precipitation with anti-RT antiserum. It can also be seen in lanes A-D (arrow, Fig.2). An env-precursor protein gPr82^{env} as well as a very small amount of p15(E) are visible in lane F. The identification of the Pr65^{gag} and gPr82^{env} proteins as Akv encoded proteins was established by hydroxylamine cleavage of these precursor proteins. By hydroxylamine cleavage closely related MLVs can be identified by the specific cleavage patterns of their precursor proteins (Saris et al., manuscript in preparation). In Fig.3 we compared the precursor proteins mentioned with precursor proteins obtained by immunoprecipitation of Akv proteins from lysates of NIH-3T3 cells that were productively infected with AKR virus. Clearly, the cleavage patterns of the gag-precursors (lanes A and B) and of the env-precursors (lanes C and D) are identical. RESA-2 cells, therefore, show normal Akv gag- and pol-precursor protein expression and processing, and expression of Akv gPr82^{env}. However, no mature gp70 is formed suggesting a lack of the necessary protease functions in these 3Y1 cells.

Based on positive RT-assays we expected that RESA-2 cells produced virions containing active RT. However, we were unable to productively infect the mouse cell lines NIH-3T3, Balb/c-3T3 or SC-1 or the mink cell line CCL-64 with these virions, suggesting that they are defective.

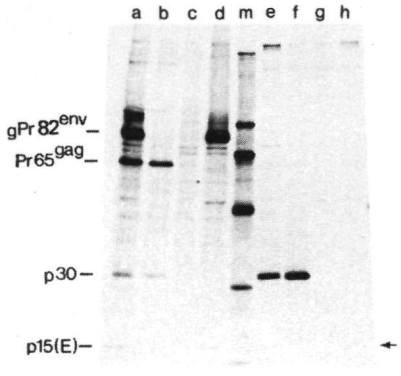

Figure 4:Protein analysis of RESA-2 virions. In order to obtain labeled virions for analysis, RESA-2 cells were labeled overnight with (^{35}S)-methionine (25 µCi/ml). After 16 hr the medium was collected and the virions were pelleted through a 20 % glycerol cushion. The RESA-2 cells were treated with trypsine as described by Van Zaane et al.(11) in order to remove any protein or parts of proteins protruding from the cell membrane. Next the cells and virions were lysed in PBSTDS and viral proteins were analysed as described in the legend to Fig.2. Lanes a-d: RESA-2 cells. Lanes e-h: RESA-2 virions. The first antisera used were in lanes a and e anti-Rauscher murine leukemia virus antiserum, lanes b and f anti-p30 antiserum, lanes c and g anti-RT antiserum and lanes d and h anti-p15(E)p12(E) antiserum.

The virion proteins were analysed and compared to viral proteins expressed in the RESA-2 cells. The result of this analysis is shown in Fig.4. From lanes a, b, c and d it can be seen that large quantities of $gPr82^{env}$ and $Pr65^{gag}$, but relatively little p30 and p15(E) are present in RESA-2 cells. Lanes e-h show the viral proteins present in the virions using the same antisera as used in lanes a-d. Lane f shows the presence of large amounts of p30, while some p15(E) is present also (lane h). However, no gp70 can be detected. We conclude that the virions do not contain mature gp70 in their envelopes and therefore are non-infectious to mouse cells. This conclusion is in agreement with other reports that gp70 itself is not necessary for budding of retroviruses (2, 3, 12). Although a low RT activity is measured in the virions we did not detect the RT (lane g), probably because the amount was too low.

RESA-2 cells contain a large number of Akv proviruses, introduced by micro-injection, and faithfully express the viral genes. However, although $Pr65^{gag}$ is processed normally we did not detect normal processing of $gPr82^{env}$ suggesting that 3Y1 cells might lack the protease function necessary for that processing. The other possibility that small deletions in the Akv genome, not detectable by restriction enzyme analysis, cause the observed defect is unlikely in view of the high number of integrated proviruses and the strong fluorescence. The lack of (a) factor(s) necessary for normal processing of $gPr82^{env}$ was further indicated when RESA-2 cells were fused with mouse cells (dense N, a subclone of NIH-3T3) that in their genome do not contain integrated proviruses of ecotropic MLVs. The virus isolated from the fusion cultures was infectious to mouse cells and displayed the characteristic host-range of AKR virus (not shown), indicating that indeed 3Y1 rat cells lack some post-integration function(s) necessary for correct processing of $gPr82^{env}$. The observed defect explains why 3Y1 cells exposed to AKR virus do not become XC- or RT-positive or contain detectable amounts of viral proteins, because the virus formed by a low number of succesful infections does not spread in the culture. The lack of $gPr82^{env}$ processing is not a general characteristic of rat cells since some rat cells (NRK for instance) are able to produce infectious virions. The high incidence of abortive infections (1, 4) must be due to the presence of many defective virions in a virus stock or to the lack of the right cellular factors required for reverse transcription and subsequent integration, or to both since abortive infections were largely prevented by micro-injection of infectious DNA.

Acknowledgements

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CHAPTER III

Expression of a subgenomic Akv DNA Fragment after Micro-injection into Mouse Balb/c-3T3 Cells

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ABSTRACT

Micro-injection of tissue culture cells was used to introduce a retroviral DNA fragment that does not contain selectable markers into Balb/c-3T3 cells. We used an 11.2 kbp fragment isolated from the DNA clone 623 that contained the 4.2 kbp 5´terminal Akv sequences. After micro-injection and screening of cultures for expression of Akv encoded proteins, three cell clones were obtained one of which (named BESA-3) was used for a detailed characterization. BESA-3 cells appeared to contain one copy of the injected fragment integrated between long terminal repeat sequences and producing a 35 S RNA related to the gag portion of Akv RNA but not packageable in pseudo virions. A slowly processed $Pr65^{gag}$ was also produced. BESA-3 cells did not synthesize $Pr180^{gag-pol}$ and did not form virions. In addition to the impaired Akv gag expression, an env related 82 kD protein was observed in immunoprecipitation studies. Restriction analysis, analysis of mRNA and precipitation reactions with monoclonal antibodies indicated that the injected fragment had recombined with endogenous non-Akv murine leukemia viral (MLV) sequences, resulting in induction of the env related protein. The defect in the rate of processing of the newly expressed $Pr65^{gag}$ (possibly associated with the loss of a restriction site in clone 623) was overcome by infection with the amphotropic 4070-A virus, BESA-3 cells being resistant to infection with ecotropic MLVs.

INTRODUCTION

Retroviral structural proteins have been studied in great detail (25). For many proteins characteristic peptide patterns or amino acid sequences

have been published. These data were confirmed by nucleic acid sequence data (23, 29). Also the relation between precursor proteins and products has been elucidated (25). However, compared to our knowledge of structural details less is known about the biological functions of these proteins. Transfection (10) and micro-injection (9) of cells in culture can be used to study biological functions. In transfection experiments the expression of retroviral DNA or of subgenomic retroviral DNA fragments (1, 16, 30) were studied. In each case the cells expressing the DNA, could be selected for. However, micro-injection can be used to assay the possible expression of a viral genomic fragment without using selectable markers, in many types of cells. Thus this approach can complement other attempts to correlate structure and biological functions including studies with ts-mutants (28, 34). In this paper we describe the introduction and expression of a subgenomic fragment of Akv DNA containing gag related sequences (13) into Balb/c-3T3 cells.

MATERIALS AND METHODS

Cells and viruses

NIH-3T3, 3Y1 and Balb/c-3T3 cells were grown in Dulbecco's modification of Eagle's medium supplemented with 10 % calf serum (GIBCO). R-MLV, M-MLV (7) and amphotropic 4070-A MLV (11) were grown in NIH-3T3 cells. AKR murine leukemia virus (further designated AKR-623) was isolated from NIH-3T3 cells productively transfected with clone 623 DNA by the calcium phosphate coprecipitation method (10). Infections of mouse cells with the viruses were performed in the presence of 4 µg/ml polybrene.

Micro-injection and selection of cells

Micro-injection was performed essentially as described by Graessmann et al.(9). DNA solutions (100 µg/ml) were introduced into nuclei of Balb/c-3T3 cells using siliconized glass capillaries with tips smaller than 0.5 µm, mounted on a Leitz micromanipulator. A culture of 100 Balb/c-3T3 cells was micro-injected and grown to confluency. Cells were examined for expression of viral proteins by an indirect immunofluorescence assay (IF) (30) using anti-R-MLV p30 antiserum (anti-p30) as the first antiserum and swine anti rabbit IgG coupled to FITC as the second antiserum (Nordic, Tilburg, The Netherlands). Cultures containing IF positive cells were cloned in microtiter plates (Falcon). Clones were screened for viral protein expression using the assay described above.

Isolation and analysis of DNA

High molecular weight DNA was isolated as described (31). Restriction enzymes EcoRI (Boehringer), SalI, XhoI, PstI, KpnI, BamHI and HindIII (Bethesda Research Labs) were used under the conditions described by the manufacturers. The digested DNA samples were separated by electrophoresis on 0.7 % agarose gels (SeaKem) and were transferred to nitrocellulose filters (Schleicher & Schüll, BA 85) by the Southern blotting technique (24). DNA fragments containing Akv sequences were detected by hybridization to cloned Akv genome fragments selected for their specificity for Akv genes (generous gift of Dr.A.J.M.Berns). Thus Akv gag and Akv env could be distinguished from genes of other MLVs (W.Quint & A.Berns, to be published).

Immunoprecipitation and SDS-PAGE

Cells to be analysed for expression of viral proteins were labeled for 15 min with ^{35}S-methionine (RCA) and were either lysed immediately or after incubation for different periods of time in chase medium (32). Viral proteins were immunoprecipitated using anti-p30 or anti-R-MLV gp70. Anti-p30 was raised in rabbits against purified p30 (32). Anti-gp70 was obtained from the NCI. Proteins were separated by electrophoresis on 7-18 % or on 10 % SDS-polyacrylamide gels. Radioactivity was visualized by scintillation autoradiography (3).

Isolation of mRNA and analysis

Total cellular RNA was isolated according to Palmiter (17). The RNA preparations were passed over oligo-dT cellulose columns (Collaborative Research,Mass.) and poly-A containing RNA was eluted from the columns in low salt buffer (2). The mRNAs were precipitated with LiCl and ethanol and dissolved in sterile TE (10 mM Tris-HCl, pH 6.8/ 1mM EDTA). Samples of the mRNA preparations were treated with glyoxal/DMSO (50 min at 50^{o}C) (15) and separated by electrophoresis on 1.4 % agarose gels. Blotting was performed according to Thomas (27).

RESULTS

Micro-injection of Balb/c-3T3 cells

Clone 623 DNA (Fig.1) was shown by transfection to harbor a biologically active Akv genome in addition to surrounding cellular sequences (13).

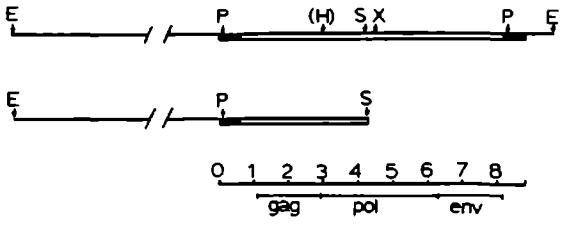

Figure 1:Map of the DNA clone 623 as determined by Rands et al.(19) and of the fragment used. Clone 623 containing Akv sequences was a generous gift of Dr.D.Lowy (13). E= EcoRI, P=PstI, H=HindIII, S=SalI and X=XhoI. The black boxes denote the long terminal repeats (LTR). The open box denotes AKR-623 DNA sequences and lines denote cellular DNA present in clone 623. The position of the viral genes on the map is based on data by Shimmick et al.(23).

The cloned DNA was digested with EcoRI and SalI and the fragments were separated on a 0.7 % agarose gel. The 11.2 kbp EcoRI-SalI fragment (Fig.1) was isolated from the gel by electro-elution and dissolved in the microinjection buffer described by Graessmann et al.(9). From the DNA concentration (100 µg/ml) and the estimated volume of the injected solution (10^{-11} ml) it is calculated that approximately 85 molecules are injected per cell. After one week the injected culture contained 5 % IF positive cells. Single cell clones were derived from the culture and, after a second screening by indirect IF, three single cell clones showed strong expression of proteins reactive with anti-p30. In a subsequent pulse labeling experiment the clones appeared to express $Pr65^{gag}$ as well as an env like 82 kD protein (based on immunoprecipitation analysis, data not shown). Because of the similarity and possible identity of the three clones the following experiments were done with one clone, designated BESA-3.

Analysis of BESA-3 chromosomal DNA

The stable expression of anti-p30 reactive proteins in the clones indicated that most probably one or more copies of the injected DNA fragment had been integrated. Furthermore, the observed expression of an 82 kD protein suggested that one of the integrated copies had induced this expression, possibly by recombination with an endogenous MLV genome. High molecular weight DNA from BESA-3 cells and from Balb/c-3T3 cells was isolated and digested with EcoRI, with EcoRI and PstI, or with HindIII. EcoRI does not cleave Akv DNA. PstI cleaves Akv DNA twice, namely in both LTRs, see Fig.1. Fragments containing Akv sequences were detected after gel separation and blotting by hybridization to an Akv specific DNA probe. The results show (Fig.2) that Balb/c-3T3 cells contain one copy of endogenous Akv (lane B) that is cleaved by PstI to give a band of 8.2 kbp

(lane D), as was shown before (18). The BESA-3 EcoRI digest, on the other hand, contains two bands hybridizing to the DNA probe (lane A). The lower band (20 kbp) represents the endogenously present Akv genome. The upper band not present in Balb/c-3T3 is apparently due to the integration of the micro-injected Akv fragment. The combined EcoRI/PstI digestions did

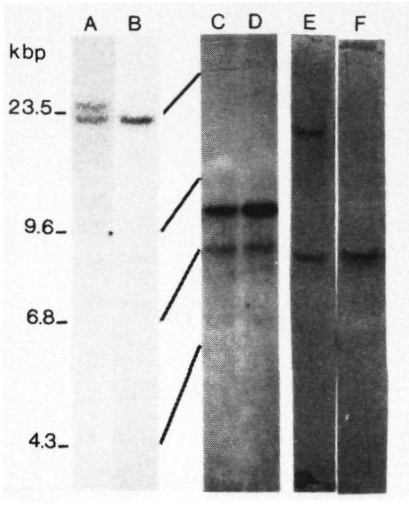

Figure 2:Analysis of the integration pattern of Akv DNA. High molecular weight DNA was isolated from BESA-3 cells (lanes A, C and E) or from Balb/c-3T3 cells (lanes B, D and F). The DNAs were digested with EcoRI (lanes A and B), with EcoRI/PstI (lane C and D) or with Hind III (lanes E and F). Hybridization was performed with an Akv specific DNA probe (lanes A-D) or with an Akv gag specific DNA probe (lanes E and F).

not reveal a difference between Balb/c-3T3 and BESA-3 (lanes D and C). The expected 8.2 kbp band is present as well as two more bands resulting from cross-hybridization with fragments of multiple copies of numerous other endogenous MLVs, that accumulate after EcoRI/PstI digestions in such amounts that they are recognized by the Akv specific DNA probe (18). The fact that no additional bands were found suggests that the micro-injected DNA fragment recombined with one of the endogenous MLVs.

These conclusions were confirmed by digestions with HindIII which recognizes a unique characteristic of the Akv 623 clone. Naturally occurring Akv proviruses all seem to posses one HindIII site at 2.8 kbp. Apparently that site was lost during the isolation of clone 623 (19). BESA-3 and Balb/c-3T3 DNAs were digested with HindIII, separated by electrophoresis and blotted. The blots were hybridized to an Akv specific probe that only detects the region from 1.8 to 2.8 kbp, i.e. to the left of the HindIII site (absent in clone 623, see Fig.1). As can be seen from Fig.2, lanes E and F, HindIII digested BESA-3 DNA contains two bands hybridizing to the probe used: one band of 14 kbp and one band of 6 kbp. In contrast, Balb/c-3T3 DNA contains only the 6 kbp HindIII fragment, resulting from cleavage of the endogenous Akv genome. The 14 kbp fragment was not detectable using an Akv env specific probe (data not shown), thus identifying the observed

band as resulting from the micro-injected DNA fragment. More evidence for a recombination of the Akv DNA fragment with an endogenous MLV comes from analysis of the 82 kD env like protein and of the mRNA present in BESA-3 cells.

Viral protein expression in BESA-3

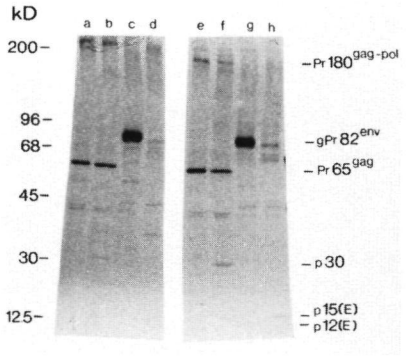

Figure 3: Characterization of viral proteins in BESA-3 cells. BESA-3 cells (lanes a-d) or NIH-3T3(AKR-623) cells (lanes e-h) were pulse labeled with ^{35}S-methionine and were lysed immediately (lanes a, c, e and g) or were chased for 6 hr (lanes b, d, f and h). Immunoprecipitations were done with anti-p30 (lanes a, b, e and f) or with anti-gp70 (lanes c, d, g and h). Molecular weight markers are indicated on the left. The immunoprecipitated proteins were separated on a 7-18 % SDS-polyacrylamide gel.

The selection of the BESA-3 line and related cell lines was based on detection of viral antigens by IF in the micro-injected Balb/c-3T3 cells. Pulse-chase experiments were performed to analyse the viral proteins in more detail. This characterization is shown in Fig.3. Viral proteins in BESA-3 cells were compared to viral proteins in NIH-3T3 cells productively infected with AKR-623 (designated NIH-3T3(AKR-623)). From precipitations of BESA-3 lysates with anti-p30 (lanes a and b) it is clear that $Pr65^{gag}$ present in the pulse lysate is still largely present after a 6 hr chase. No $Pr180^{gag-pol}$ is observed in these lysates. However, a small but detectable amount of p30 is present in the chase lysate, indicating that $Pr65^{gag}$ is processed to some extent in BESA-3 cells despite the fact that they do not produce virus particles (not shown). A similar slow processing of $Pr65^{gag}$ and also of $Pr180^{gag-pol}$ was observed in lysates of NIH-3T3(AKR-623) cells (lanes e and f). A significant amount of $Pr65^{gag}$ is still present in 6 hr chase lysates, whereas for other MLVs the processing of $Pr65^{gag}$ is normally complete after 2-3 hr (see for instance ref. 32). On the other hand, in NIH-3T3(AKR-623) cells the processing of the $gPr82^{env}$ precursor protein is quite normal in that after a 6 hr chase the mature envelope proteins gp70, p15(E) and p12(E) are visible as well as the precursor $gPr82^{env}$ (lane h) as was shown for many other strains of MLV (e.g. ref.32).

Immunoprecipitations with anti-gp70 reveal the presence of an 82 kD protein in pulse lysates of BESA-3 cells (lane c) that disappears during the 6 hr chase, but does not give rise to known mature env proteins (lane d). Also the 82 kD protein could not be precipitated with anti-R-MLV-p15(E)p12(E) antiserum or with monoclonal anti-Akv-gp70 or anti-Akv-p15(E) antisera (data not shown). Further, in contrast to the $gPr82^{env}$ in NIH-3T3(AKR-623) cells the 82 kD protein in BESA-3 cells is not cleaved by hydroxylamine, a characteristic found thus far only for xenotropic MLV (C.J.M.Saris, unpublished observation). IF studies indicated that the protein is located in or close to the cell membrane (not shown), a pattern characteristic for env precursor proteins of some xenotropic and dualtropic MLVs (6).

mRNA in BESA-3 cells

The efficient expression of the 82 kD protein together with the DNA restriction analysis suggested that in one of the cells the micro-injected 11.2 kbp fragment recombined with an endogenous MLV genome, thereby facilitating its stable integration and expression.

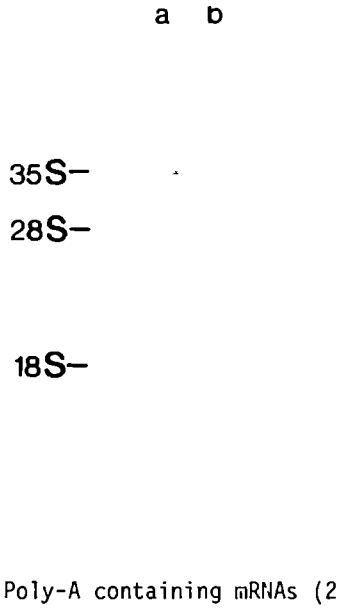

Figure 4: Analysis of mRNA containing Akv sequences. mRNAs were isolated from BESA-3 cells (lane a) as described and were separated on an agarose gel after treatment with glyoxal/DMSO. For comparison virion RNA was isolated from AKR-623 and run on the same gel (lane b). After blotting the filter was hybridized to a plasmid containing 5´end Akv specific DNA sequences. 35 S virion RNA and ribosomal 28/18 S RNA were run as markers.

If this suggestion is correct the sizes of the viral mRNA present in BESA-3 cells should be identical to those of MLV infected cells, a 35 S species coding for gag and pol precursor proteins and a 22 S species coding for the env precursor protein, and they should contain Akv sequences originating from the 5´end of AKR-623.

Poly-A containing mRNAs (2) were isolated from BESA-3 cells and treated with glyoxal/DMSO . After separation by electrophoresis on an agarose gel and blotting onto a nitrocellulose filter the RNAs were hybridized to a plasmid containing sequences derived from gag encoding sequences of the 5´

end of AKR-623. Fig.4 shows a comparison of BESA-3 mRNA (lane a) with virion RNA isolated from AKR-623 virus preparations (lane b). It is clear that the BESA-3 cells contain a 35 S mRNA harboring 5´Akv sequences. Furthermore, we were unable to detect the 35 S species or a 22 S species using an env Akv specific probe (data not shown), thus adding support to the proposed recombination event.

Kinetics of $Pr65^{gag}$ processing in BESA-3 cells and in NIH-3T3(AKR-623) cells

We observed that AKR-623 grows to low titers and that the processing of its precursor proteins $Pr65^{gag}$ and $Pr180^{gag\text{-}pol}$ is very slow compared to proteins of other MLVs (see Fig.3). Thus far, the only difference found between AKR-623 DNA and proviral Akv DNA is the loss of the HindIII site at 2.8 kbp in AKR-623 DNA (see Fig.1, (19)). This site is located approximately at the junction of the gag and pol genes. Three possibilities arise: a mutation in one of the proteins encoded in this arrea might impair a hypothetical protease function; alternatively, the mutation might impair the induction of a cellular protease after infection of cells with AKR-623 or, finally, the mutation might have caused a change in one of the protease recognition sites resulting in inefficient cleavage. If either of the first

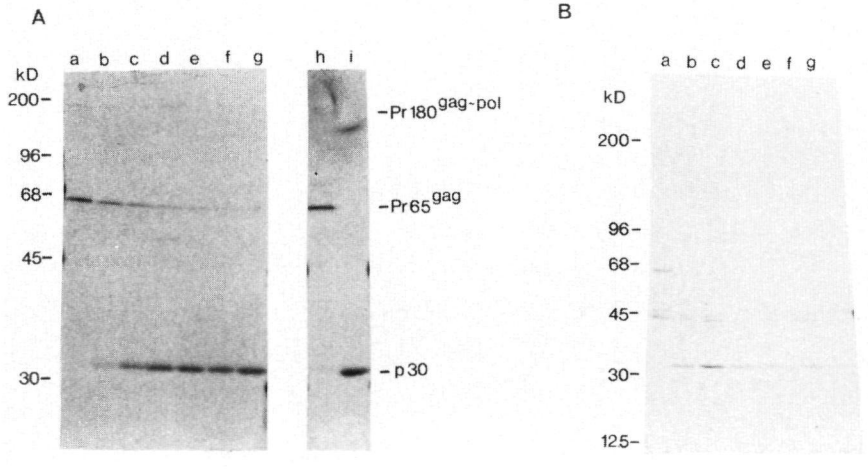

Figure 5:Processing of AKR-623 $Pr65^{gag}$. (A) NIH-3T3(AKR-623) cells (lanes a-g) or NIH-3T3(4070-A) cells (lanes h and i) were pulse labeled with ^{35}S-methionine and were lysed immediately (lanes a and h) or were chased for 1 hr (lane b),2 hr (lanes c and i), 3 hr (lane d), 4 hr (lane e), 5 hr (lane f) and 6 hr (lane g). Immunoprecipitations were done with anti-p30. (B) BESA-3(4070-A) cells were pulse labeled and chased under conditions as described for the NIH-3T3(AKR-623) cells in section (A).

two alternatives were true, then the processing of $Pr65^{gag}$ in BESA-3 cells should be normal after infection of these cells with another MLV that would provide or induce the presumed protease function. We failed to infect BESA-3 cells with ecotropic MLVs: no unintegrated viral DNA was found after infection of BESA-3 cells, although we found that the ecotropic MLVs used still absorbed to the cell membrane (not shown). Next we tried infections with an amphotropic MLV, 4070-A, and obtained BESA-3 cells productively infected with 4070-A.

The processing of $Pr65^{gag}$ was compared between BESA-3(4070-A), NIH-3T3(AKR-623) and NIH-3T3(4070-A). Cells were pulse labeled and subsequently chased for the indicated periods of time., ranging from 1 to 6 hr. From Fig.5A it can be seen that the $Pr65^{gag}$ and $Pr180^{gag\text{-}pol}$ processing in NIH-3T3(4070-A) cells is normal (lanes h and i), whereas the processing of these proteins in NIH-3T3(AKR-623) cells is slow and incomplete even after 6 hr (lanes a-g). In BESA-3(4070-A) cells the processing of both gag precursor proteins is as efficient as in NIH-3T3(4070-A) cells (Fig.5B). Therefore, 4070-A encoded proteins or 4070-A induced cellular proteins can efficiently process the Akv $Pr65^{gag}$.

Lack of packaging of Akv RNA in 4070-A virions in BESA-3(4070-A) cells

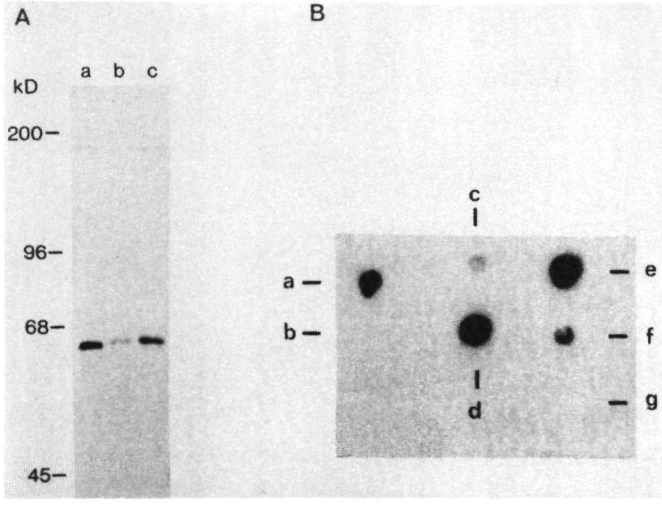

Figure 6: Analysis of virus produced by BESA-3(4070-A) cells. (A) $Pr65^{gag}$ from three sources: NIH-3T3 cells were infected with virus from NIH-3T3(AKR-623) cells (lane a), from BESA-3(4070-A) cells (lane b) or from NIH-3T3(4070-A) cells (lane c). After three passages the infected cells were pulse labeled with ^{35}S-methionine and lysed. Viral proteins were immunoprecipitated with anti-p30. (B) Akv RNA: mRNA (0.4 µg) or virion RNA (0.04 µg) isolated from the cell lines indicated below were spotted on a nitrocellulose filter and hybridized to the plasmid, containing Akv gag specific sequences. (a) BESA-3 mRNA, (b) BESA-3 mRNA 1/10 amount used in (a), (c) mRNA from NIH-3T3(AKR-623), (d) mRNA from 3Y1(AKR-623), (e) AKR-623 virion RNA, (f) AKR-623 virion RNA 1/10 amount used in (f), (g) BESA-3(4070-A) virion RNA.

The infection of BESA-3 cells with 4070-A opened the possibility to investigate whether or not the 35 S Akv specific RNA (Fig.4) in BESA-3 cells could be encapsidated in type C virions. The answer was negative. In experiments in which infections were performed of NIH-3T3 cells with virus released from BESA-3(4070-A) cultures, we only observed expression of the 4070-A $Pr65^{gag}$ precursor protein, as is shown in Fig.6A. The Akv $Pr65^{gag}$ and 4070-A $Pr65^{gag}$ have a slightly different molecular weight on 10 % SDS polyacrylamide gels (lanes a and c). Therefore it was of interest to determine whether mRNA, containing Akv sequences, is packaged in virions released from BESA-3(4070-A) cells. RNA was isolated from virions released from NIH-3T3 (AKR-623) and from BESA-3(4070-A) cultures and was spotted on a nitrocellulose filter together with mRNA from BESA-3, NIH-3T3(AKR-623) and rat cells (3Y1) expressing AKR-623. Fig.6B shows the results after hybridization of the filter to the described plasmid, containing Akv gag specific sequences. The probe does not detect M-MLV, M-MSV, R-MLV or 4070-A RNAs (data not shown). It can be seen that virions from BESA-3(4070-A) cells (spot g) do not contain detectable amounts of Akv RNA, using the same amounts in comparison with virion RNA from AKR-623 (spots e and f). Spots containing 1/100 the amount of Akv RNA used in spot e can be detected (not shown). Therefore, Akv RNA sequences in BESA-3(4070-A) cells are not packaged as pseudotypes to a significant extent (less than 1 %).

DISCUSSION

Micro-injection of DNA fragments

The results described in this report demonstrate that micro-injection of retroviral fragments can be used to stably introduce such fragments in chromosomal DNA of cells. In the case of the fragment used (Fig.1) only the 5'LTR is present, whereas during retrovirus infections an intermediate containing two LTRs is found integrated in a specific way in the chromosomal DNA. Single cell clones could be selected for using IF. Therefore, in some cases micro-injection might be preferred over transfection when no selectable markers are expressed by the particular DNA fragment. The DNA is introduced without the addition of carrier DNA (33). Transfections with DNA clones without the addition of carrier DNA appear to occur at low efficiences relative to the same transfections performed in the presence of carrier DNA. We observed approximately 5 % IF positive cells after micro-injection and we isolated three cell clones from this culture that proved to be identical on the basis of restriction analysis and protein analysis.

They contained only one copy of the injected fragment, although the cells were injected with approximately 85 molecules each. The mRNA and DNA analyses in which several Akv specific gag or env DNA probes were used, suggest that the stable expression of the fragment was facilitated by a recombination event with endogenous MLV sequences.

AKR-623 is mutated in its $Pr65^{gag}$ processing

The cell line described expresses newly introduced Akv sequences originating from the gag region of AKR-623. A $Pr65^{gag}$ precursor protein is observed that is indistinguishable from $Pr65^{gag}$ synthesized in NIH-3T3(AKR-623) cells. Recently it was published that AKR-623 differs only from the parental Akv by the absence of the HindIII site at 2.8 kbp (19). The complete base sequence of M-MLV has been published (23). The location and size of the gag and pol genes of all MLVs are presumably almost identical (Fig.1). Therefore, the HindIII site can be placed in the vicinity of the gag-pol junction. The loss of the HindIII site (resulting either from a point mutation or from a small deletion) could exert influence on either side of this junction. The experiments presented in Fig.5 show that the mutation did not cause a change in the junctions between the mature gag proteins or between gag and pol such that they are not recognized efficiently by a protease, because in BESA-3(4070-A) cells $Pr65^{gag}$ is cleaved efficiently. Also the processing of Akv $Pr65^{gag}$ can be accomplished by a non-related MLV (4070-A). It was shown before that the processing of Simian sarcoma virus coded $p65^{gag}$ is efficient after infection of non-producer SiSV cells with the related SiSAV (26). Also it was shown that the $p65^{gag}$ coded by Gazdar murine sarcoma virus can be cleaved in vitro by related, detergent disrupted, M-MLV (14). Therefore, the results suggest that AKR-623 is mutated in its protease function or in its ability to induce this function.

Interference in BESA-3 cells for ecotropic MLVs

In the course of this study we noted that BESA-3 cells could not be productively infected with ecotropic MLVs, whereas infections with an amphotropic MLV succeeded. This was rather unexpected because firstly, no virus is released from BESA-3 cultures that might interfere with ecotropic MLVs (22), and secondly, although an envelope polyprotein is expressed in BESA-3 cells this alone is not sufficient to explain the absolute interference observed (4). Indirect-IF data suggest that the 82 kD protein is located close to or within the cell membrane, a feature

characteristic for env precursors of xenotropic or amphotropic MLVs (5). Furthermore, the absence of mature env proteins in BESA-3 cells, the failure to precipitate the 82 kD protein using anti-p15(E)p12(E) or Akv env specific monoclonal antisera and the disappearance of this protein in chase lysates suggest a resemblance to Moloney Cell Surface Antigen, a protein related to xenotropic env proteins (12). More detailed studies using labeled virus revealed that ecotropic MLVs can still absorb to the cell membrane, but do not penetrate into the cytoplasm (not shown). It is unclear if the 82 kD protein interferes with this penetration.

Akv RNA is not packaged in virions released from BESA-3(4070-A) cells.

It is not yet clear what rules must be obeyed by RNA species to be efficiently packaged in type C virions. Some evidence suggests that RNA should be able to form a 50-70 S complex (21) and that a particular 5' sequence, spliced out in viral mRNAs, has to be present (20). Of course, when RNA should not only be packaged but also be faithfully reversily transcribed, redundant sequences located at the 3'end in retroviral genomes have to be present (8, 21). The restriction analysis of BESA-3 chromosomal DNA, the presence of a 35 S mRNA containing Akv gag but not Akv env sequences and the efficient expression of the non-Akv 82 kD env related protein suggest that the injected fragment had recombined with one of the multiple copies of endogenous MLVs. Therefore we expected the RNAs transcribed from these sequences to be packaged as 4070-A pseudotypes and to be transferable to NIH-3T3 cells. However, experiments designed to prove this, showed that no RNA species, containing Akv sequences, were packaged as pseudotypes to a detectable degree.

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Biochemistry

Molecular cloning of murine endogenous viral sequences and expression of a newly constructed recombinant murine leukemia virus DNA in transfected mink cells

(recombinant DNA/restriction endonuclease mapping)

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ABSTRACT **In the process of molecularly cloning unintegrated proviral DNA from NIH-3T3 mouse cells infected with Rauscher murine leukemia virus, we observed the occurrence of clones with inserts smaller than the expected Rauscher murine leukemia virus fragments. The insert of one of these clones, λ Xe-1, was characterized in more detail. It had a size of 3.5 kilobases. The restriction map was similar but not identical to that of the envelope regions of Moloney and Rauscher murine leukemia viruses. After ligation to previously cloned Moloney murine leukemia viral sequences and transfection of the ligated DNA into mink lung cells, a nondefective xenotropic murine leukemia virus, XH-19, was isolated. Restriction mapping of proviral DNA isolated from mink lung cells chronically infected with XH-19 showed the presence of Moloney murine leukemia virus-derived sequences coupled to xenotropic viral sequences.**

Some groups of viruses contain large numbers of closely related species. This is certainly true for the retroviruses. For instance, among the mammalian type C viruses there is crossreactivity in immunoprecipitations of viral proteins and in nucleic acid hybridizations. The mouse genome contains scores of complete or incomplete endogenous type C proviruses; how many different types there are is not known (1).

The development of viral leukemia in mice is accompanied by multiple recombinations and reintegrations of viral genomes (2). The replication defective transforming type C viruses arose from recombinations between the genomes of replication competent viruses and certain cellular sequences (3). Precise analyses of recombinant viruses are undertaken to gain insight in the process of leukemogenesis (2) or in the potential oncogenicity of certain cellular sequences (4). Another experimental approach would be the construction of recombinant viruses *in vitro* as made feasible by a combination of modern techniques, particularly the molecular cloning of DNA and transfer of DNA to cultured cells (transfection).

In this paper we report the construction *in vitro* and characterization of a recombinant virus derived from complementary parts of the genomes of an ecotropic and xenotropic murine leukemia virus.

MATERIALS AND METHODS

Cell Lines and Viruses. The cell lines used were the mouse cell lines NIH 3T3, BALB/c 3T3, NCL5611P3 (an NIH-3T3-derived subclone), and SC-1 rat cell lines F-111 and 3Y1 and the mink lung cell line CCL-64. The ecotropic virus used to generate circular viral DNA was a clonal isolate wt-248 of Rauscher murine leukemia virus (R-MLV) (5). Moloney murine leukemia virus (M-MLV) (clone A) was grown in NIH-3T3 cells. Xenotropic and dualtropic viruses were grown in CCL-64 cells and Akv-2 was grown in SC-1 cells.

DNA Isolation Procedures. Nonintegrated viral DNA was isolated from NIH-3T3 cells by the Hirt procedure (6) 16 hr after infection with wt-248 R-MLV isolated from productively infected NCL5611P3 cells. High molecular weight DNA was isolated as described (7).

Cloning. Closed circular R-MLV DNA isolated from a low molecular weight DNA preparation was digested with *Hin*dIII, which cleaves R-MLV DNA at two sites. The resulting fragments were joined to *Hin*dIII restricted λ Charon 21A DNA with phage T4 ligase. The protocol of Blattner *et al.* (8) was used for *in vitro* packaging of the ligated DNA into infectious λ particles. Resulting recombinant clones were screened with a representative R-MLV cDNA probe (7). Plaqued viruses hybridizing with this probe were examined for the size of the inserted DNA fragment. All experiments were performed in accordance with the National Institutes of Health guidelines for P2 laboratory facilities with certified EK2 or EK1 CV vectors. The origin of the recombinant clone λ MLV_{int}-1 was described elsewhere (9).

Restriction Endonuclease Analysis and Ligation. DNA preparations were digested with commercially available restriction enzymes and were analyzed on agarose gels ranging between 0.7% and 1.2%. The restriction fragments were identified by hybridization with a labeled cDNA probe after transfer onto nitrocellulose filters by the Southern blotting technique (10). For ligation experiments the λ MLV_{int}-1 and λ Xe-1 DNAs were cleaved with *Hin*dIII. After separation on 0.7% agarose gels the DNA inserts [8.8 and 3.5 kilobases (kb), respectively] were electro-eluted as described (7). One microgram of the 8.8-kb insert was ligated to 3 μg of the 3.5 kb insert by using T4 DNA ligase.

Transfection. Samples of the ligation products (500 ng each) were used to transfect CCL-64 cells and NIH-3T3 cells using the calcium phosphate coprecipitation method (11). After three passages sedimentable reverse transcriptase activity indicated the presence of a virus in the medium of the transfected CCL-64 cells. The virus was named XH-19.

Characterization of the Virus XH-19. (i) *DNA.* High molecular weight DNA from CCL-64 cells productively infected with XH-19 was isolated and cleaved with several restriction en-

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Abbreviations: kb, kilobase(s); LTR, long terminal repeat; M-MLV, Moloney murine leukemia virus; R-MLV, Rauscher murine leukemia virus.

zymes After separation on agarose gels and Southern blotting fragments were detected by hybridization to an M MLV specific cDNA probe (2)

(ii) *Viral proteins* NIH 19-producing CCL 64 cells were labeled with [^{35}S]methionine in typical pulse–chase experiments as described 12) and virus specific polypeptides were detected by using rabbit antiserum directed against detergent disrupted R-MLV (anti-R MLV) against R MLV envelope glycoprotein gp70, or against R MLV envelope protein p15(E) Immunoprecipitated proteins were separated on sodium dodecyl sulfate/8–17% polyacrylamide gels as described (12)

(iii) *Host range* Infectivity of the NIH 19 virus was tested on several cell lines after infection in the presence of Polybrene at 4 μg/ml After 2 days the cells were washed twice with phosphate buffered saline and fixed in acetone for 10 min at room temperature Indirect immunofluorescence tests were performed as indicated below (see iv) using a 1 50 dilution of a broadly reactive antiserum raised against Tween disrupted M MLV virions (anti-M-MLV)

(iv) *gp70 and p15(E) antigenic determinants* Indirect immunofluorescence experiments were performed by the method of O Donnell and Nowinski (13) Briefly cells were allowed to attach to multispot immunofluorescence slides overnight and were fixed in acetone for 10 min at room temperature The ascites fluid containing monoclonal antibodies 19-A2 16-C1 16 B7 9 E8 19 VIII E8 or 19 F8 (kindly provided by R C Nowinski) was applied at a dilution of 1 100 in phosphate buffered saline Tissue culture supernatant containing the monoclonal antibody 24-6b was kindly provided by J I Portis and was used undiluted After 45-min incubation at room temperature the slides were washed in phosphate buffered saline dried and incubated for 45 min with a fluorescein isothiocyanate-conjugated goat-anti mouse Ig serum (6M17 01 F01)

Immunofluorescence was determined with a Leitz fluorescence microscope

RESULTS

Generation of Clone λ Xe-1 NIH 3T3 cells were infected with R MLV isolated from productively infected NCL5611P3 cells and 16 hr after infection viral DNA was extracted from the cells by the Hirt method Closed circular DNA was isolated from this DNA by separation on an agarose gel The following forms of unintegrated proviral DNA were present in this preparation one form of closed circular DNA (I) two forms of nicked circular DNA (II) and linearized DNA (III) of 8 8 kb [two long terminal repeats (LTRs) see Fig 4*B*] (results not shown) The circular DNA was cleaved with *Hin*dIII yielding two fragments the larger form of circular DNA (two LTRs) generates fragments of 4 7 and 4 1 kb whereas the smaller form (one LTR) generates two fragments of equal size 4 1 kb (results not shown) The presence of two LTRs in linear proviral DNA and either one or two in circular proviral DNA of retroviruses is well documented (e g , refs 9 14 and 15) A portion of the *Hin*dIII cleaved DNA was cloned in λ Charon 21A and plaques were screened with a representative R MLV cDNA probe Two clones appeared to contain inserts smaller than 4 1 kb One of these clones λ Xe-1, contained a 3 5 kb insert that poorly hybridized with the R-MLV cDNA probe suggesting that it was derived from a MLV other than the Rauscher strain

When large scale preparations of λ Xe 1 were made we observed a deletion in the insert DNA of a fraction of the recombinant molecules (Fig 2 lane a) Digestion of the relatively pure 3 5-kb insert with the enzymes *Pst* I *Kpn* I or *Bam*HI (Fig 1*A*) revealed that none of the restriction sites was removed as a consequence of the deletion

In an overexposure of the blot shown in Fig 1*A* the presence

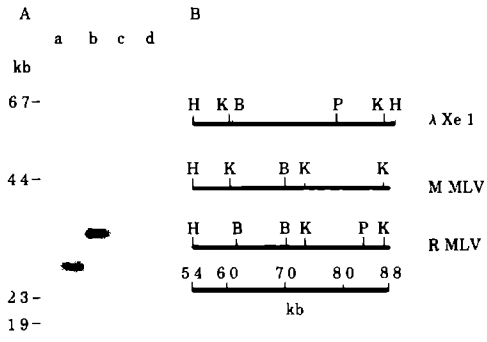

FIG 1 Restriction endonuclease analysis of the 3 5 kb insert in λ Xe 1 (*A*) λ Xe 1 was first digested with *Hin*dIII and then digested with *Pst* I (lane a) *Bam*HI (lane b), *Bam*HI and *Kpn* I (lane c) or *Kpn* I (lane d) After separation on a 1 0% agarose gel the fragments were detected by using a labeled M MLV cDNA (*B*) Physical map of the 3 5 kb insert from λ Xe 1 The map is aligned with comparable regions of M MLV (14) and R MLV (unpublished results) H *Hin*dIII, K *Kpn* I B *Bam*HI P *Pst* I

of the smaller insert was visible From this it could be seen that the *Pst* I 2 6 kb fragment did not have a shortened counterpart whereas the 2 6 kb *Kpn* I or the 3 0 kb *Bam*HI fragments did have smaller related fragments the size of the deletion is approximately 0 4 kb More double digestions were performed (data not shown) From these data the physical map was constructed The deletion could be localized between the *Pst* I site and the right *Kpn* I site in the insert DNA (Fig 1*B*) The mapping of the deletion is important for the characterization of the ligation products described in the following section

In Fig 1*B* the restriction map of the insert in the λ Xe 1 clone is positioned with respect to the comparable region in the genomes of M MLV (14) and R MLV (unpublished results) so as to obtain optimal alignment of restriction sites relative to the *Hin*dIII site From these data it was reasoned that the isolated 3 5 kb insert DNA might represent the 3′ end of a RNA tumor virus present in NIH-3T3 cells and picked up in the cloning procedure

***In Vitro* Construction of a Recombinant Viral Genome** The λ MLV$_{int}$-1 DNA was characterized previously (9 It contains an insert of 8 8 kb with cellular as well as M MLV genetic sequences The virus specific genetic sequences are 5 4 kb in length and contain the 5′ end of the integrated M MLV genome up to the *Hin*dIII site shown on the left-hand side of the map in Fig 1*B*

This insert of the λ MLV$_{int}$-1 clone was isolated and ligated to bacterial alkaline phosphatase-treated 3 5/3 1-kb insert DNAs from the λ Xe 1 clone In Fig 2 lane b it is shown that after ligation the 8 8 kb insert DNA is linked to the 3 5-kb as well as to the 3 1-kb insert DNAs resulting in two classes of molecules of 12 3 kb and 11 9 kb Thus the doublet at 12 3 and 11 9 kb in lane b proves that the intended ligation did indeed occur

We anticipated that part of the ligation products would contain the necessary information in the right configuration for infectious virus production Therefore portions of the ligation products were used to transfect NIH 3T3 cells and CCL 64 cells None of three transfection experiments of the ligated

FIG 2 Characterization of ligation products Inserts from λ Xe-1 and λ MLV_{int} 1 were isolated and ligated by using T4 DNA ligase A sample was run on a 0 7% agarose gel and DNA was detected after Southern blotting transfer by hybridization to labeled M MLV cDNA Lane a shows the 3 5-kb insert and its shortened counterpart, lane b, ligation products

DNA on NIH-3T3 cells gave rise to production of infectious virus Two transfections of C CL 64 cells however resulted in virus production after three passages of the transfected cultures as monitored by the presence of sedimentable reverse transcriptase activity in the culture fluid

Characterization of the Recombinant Virus XH-19. In order to show that the newly constructed virus XH 19 does indeed contain M-MLV sequences and to characterize its envelope proteins and host range the following analyses were performed

(*i*) The host range of XH-19 was determined by using cell lines derived from several animal species Anti M MLV antiserum was applied in the indirect immunofluorescence assay From the results of this test it could be seen that none of the mouse cell lines used (NIH 3T3 BALB/c-3T3 and SC-1 or rat cell lines used (F 111 and 3Y1 was susceptible to infection with XH-19 Only the mink lung cell line C C L-64 could be infected The pattern is characteristic for xenotropic viruses in contrast with ecotropic viruses which infect mouse cells but no cells of other species, as well as in contrast with dualtropic viruses, which infect both mouse and mink cells

(*ii*) The antigenic determinants of the envelope proteins *env* gene products see Fig 4*B*) were more directly characterized by using monoclonal antibodies directed against gp70 or p15(E) determinants In the assay shown in Table 1 we compared XH 19 with several other viruses among which were representatives of the ecotropic dualtropic and xenotropic classes In these tests, none of the antisera that are reactive against ecotropic determinants on gp70 gave a positive immunofluorescence either with the xenotropic virus AKR 219 or with XH 19 Also the lack of reaction with 9-E8 and the positive reaction with 24-6b indicate that the envelope proteins of XH-19 display a typical xenotropic character

(*iii*) Viral proteins were studied in CCL-64 cells infected with XH-19 by labeling with [^{35}S methionine for 20 min Then the cells were lysed either immediately or after incubation for another 6 hr in chase medium Virus-specific proteins were precipitated from the lysates by incubating first with anti-R-MLV anti-R-MLV gp70 or anti-R-MLV p15(E) antiserum and then with staphylococcal protein A-Sepharose The antisera used detect interspecies specific determinants The immunoprecipitated proteins were separated on sodium dodecyl sulfate/polyacrylamide gels as shown in Fig 3

As can be seen in Fig 3A anti R MLV precipitated gp70 and p15(E) as well as the *gag* gene product p30 in XH-19 chase lysates Also small amounts of $gPr76^{env}$ [the precursor of gp70 and p15(E)] and $Pr65^{gag}$ (the precursor of p15 p12 p30 and p10) are still present after 6-hr chase (lane 7) The antiserum does not recognize p12 p10 and p15 are not seen because they do not contain methionine For a comparison AKR MCF-247-infected SC-1 cells were included Lane 8 (Fig 3A) shows gp70, p15(E and p30 in the MCF chase lysate A clear difference in molecular weight is visible for these three proteins when they are compared to the XH-19 proteins Also a large amount of $gPr76^{env}$ is still present in the MCF chase lysate after 6 hr indicative of the slow processing of this protein, as was shown before by Famulari and Jelahian (17) In this figure the identity of the viral proteins precipitated with the polyvalent anti R-MLV antiserum is shown in a parallel precipitation with anti R-MLV gp70 (lanes 1–6) From lanes 1–3 it can be seen that the molecular weights of $gPr76^{env}$ are the same for XH-19 M MLV and AKR MC F 247 As a negative control lane 6 shows the immunoprecipitation of proteins in labeled lysates of uninfected CCL 64 cells no viral proteins can be detected

Because the 3 5 kb insert was isolated in a cloning experiment in which R-MLV DNA was present we performed an additional test to exclude the possibility of contamination by R-MLV in the virus preparations obtained after transfection For this purpose the proteins of R MLV and of XH-19 preparations were compared in pulse–chase experiments (Fig 3B) Lanes 1 and 5 show negative controls from uninfected NIH-3T3 cells The pulse experiments shown in lanes 2–4 reveal that the R-MLV $gPr82^{env}$ has a molecular weight clearly higher than that of the XH-19 $gPr76^{env}$ Moreover the gp70s and p15(E)s of XH

Table 1 Immunofluorescent staining of the cytoplasm of infected cells by monoclonal anti MLV antibodies

	Immunofluorescence with antibodies*						
Virus	19 A2 (gp70^c)	16 C1 (gp70^b)	16-B7 (gp70^a)	9 E8 [p15(E)^a]	19 VIIIE8 [p15(E)^b]	19-F8 [p15(E)^c]	24-6
R MLV	+	–	–	+	–	–	–
M-MLV	–	–	–	+	–	–	–
Akv 2	+	+	+	–	–	+	–
AKR MCF 247	+	+	–	+	+	+	+
AKR 219	–	–	–	–	+	+	+
XH 19	–	–	–	–	+	+	+

* Nomenclature described in detail by Lostrom *et al* (16)

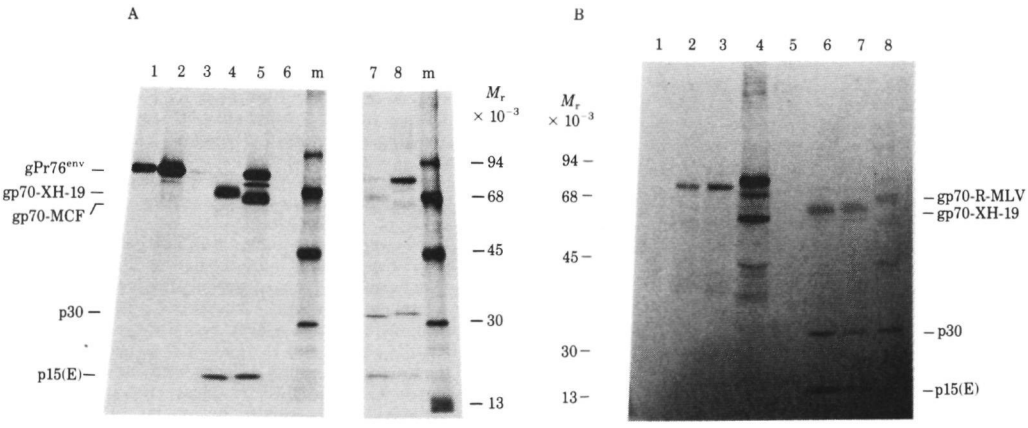

FIG. 3. Identification of XH-19-coded viral proteins. The gp70 proteins are identified by source. (*A*) CCL-64 cells infected with XH-19, SC-1 cells infected with AKR MCF-247, and NIH-3T3 cells infected with M-MLV were pulse labeled with [^{35}S]methionine for 20 min and immediately lysed or cultured for another 6 hr in the absence of [^{35}S]methionine and then lysed. Viral proteins were immunoprecipitated with anti-R-MLV gp70 (lanes 1–6) or with anti-R-MLV (lanes 7 and 8) and analyzed by sodium dodecyl sulfate/polyacrylamide gel electrophoresis and scintillation autoradiography. Lane 1, pulse, XH-19; lane 2, pulse, AKR MCF-247; lane 3, pulse, M-MLV; lane 4, chase, XH-19; lane 5, chase, AKR MCF-247; lane 6, chase, uninfected CCL-64 cells; lane 7, chase, XH-19; lane 8, chase, AKR MCF-247. The lanes marked m show marker proteins. (*B*) For a comparison between XH-19 and R-MLV, cells were labeled for 20 min with [^{35}S]methionine and lysed immediately (lanes 1–4) or after a 6-hr chase period (lanes 5–8). Viral proteins were immunoprecipitated with anti-R-MLV. Lanes 1 and 5, uninfected NIH-3T3 cells; lanes 2 and 3 and lanes 6 and 7 show labeling experiments for two independent XH-19 isolates. Lanes 4 and 8, NIH-3T3 cells infected with wt-248 R-MLV.

19 and R-MLV are clearly different, as seen in the chase experiments (lanes 6–8).

Also, the patterns obtained after cleavage of the envelope precursor proteins with hydroxylamine are markedly different (C. J. M. Saris, personal communication). Furthermore, the R-MLV gp70 migrates slower than the XH-19 gp70 (lanes 6–8), whereas the R-MLV p15(E) seems to migrate somewhat faster than the XH-19 p15(E).

(*iv*) In order to show that the xenotropic virus XH-19 contains M-MLV sequences, high molecular weight DNA was isolated from CCL-64 cells productively infected with XH-19. The DNA was digested with enzymes that were expected to cleave XH-19 DNA at sites that would generate characteristic internal fragments. Digestions were performed with *Pst* I, *Bam*HI, *Xba* I, *Xho* I, *Kpn* I, and *Hin*dIII or combinations of these enzymes. Of the resulting digests, three are shown in Fig. 4*A*. In lane c the *Pst* I digest contains a major band of 6.7 kb, which can result only from cleavage at the M-MLV site at 1.15 kb (15) and at the xenotropic viral site at 7.9 kb (see Fig. 4*B*). This result was confirmed by using *Bam*HI (lane a), which generates a 2.2-kb band resulting from cleavage at the M-MLV site at 4.05 kb (15) and at the xenotropic viral site at 6.2 kb (see Fig. 4*B*). Lane b shows a major 5.6-kb band in the *Xba* I digest, which results from cleavage at the M-MLV site at 0.28 kb (15) and at the xenotropic viral site at 5.9 kb (Fig. 4*B*), indicative of the recombinant nature of the virus XH-19.

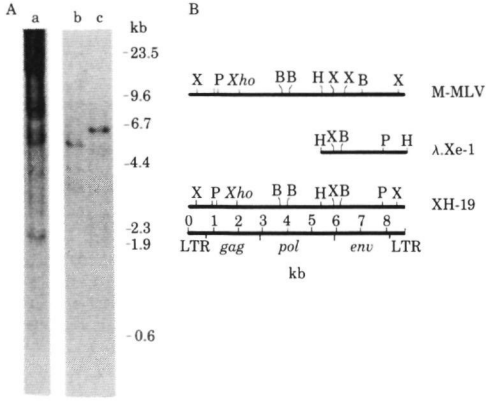

FIG. 4. Characterization of the recombinant nature of XH-19. (*A*) High molecular weight DNA isolated from CCL-64 cells infected with XH-19 was digested with *Bam*HI (lane a), *Xba* I (lane b), or *Pst* I (lane c). After separation on 0.7% agarose gel the fragments were transferred by the Southern blotting technique and detected with a labeled M-MLV cDNA made specific for M-MLV sequences. (*B*) Comparison between the physical maps of M-MLV (14), the insert of the clone λ.Xe-1, and XH-19. X, *Xba* I; P, *Pst* I; B, *Bam*HI; H, *Hin*dIII.

The presence of an *Xba* I site at 0.28 kb in the XH-19 proviral DNA indicates that the new virus acquired LTRs at both sides in the provirus derived largely from M-MLV.

DISCUSSION

We cloned a 3.5-kb *Hin*dIII fragment that apparently contained unknown viral sequences. It was recognized by a murine leukemia virus cDNA probe. With the 3.4-kb 3′-terminal *Hin*dIII fragment of linear proviral DNA of M-MLV it shared two *Kpn* I sites, one of which lies in the LTR of both M-MLV and R-MLV (Figs. 1 and 4), and one *Xba* I site (Fig. 4). Later experiments indicated the presence of a xenotropic *env* gene in the clone. Therefore, the recombinant clone was called λ.Xe-1. Its insert was picked up from a Hirt supernatant prepared 16 hr after infection of NIH-3T3 cells with R-MLV. Hence, it could have been derived from NIH-3T3 chromosomal sequences present in the Hirt supernatant. Alternatively, endogenous viral RNA sequences possibly induced after infection of the NIH-3T3 cells

with R-MLV might have become converted into proviral DNA after infection with R-MLV. This, however, seems less likely first because a cloned isolate of R-MLV (wt 248) was used and second because the NIH-3T3 cells do not seem to contain inducible endogenous viruses (1). A comparison with two DNA clones [14.9 and 8.13 (R. Mural, personal communication)] containing endogenous viral sequences which were both derived from NIH 3T3 cellular DNA showed a very large degree of similarity, supporting the first possibility.

Two successive large scale preparations of λ Xe-1 consisted partly of molecules with a 0.4 kb deletion in the putative LTR of the insert. LTRs and their flanking sequences are prone to deletion events when propagated in phage DNA as a vector (15) because of their resemblence to insertion sequence (IS) elements in transposons (18, 19). Transposons can lose their IS elements and part of the flanking sequences when taking part in recombination events (20). Therefore, the independent occurrence of the same deletion at a site corresponding to the 1 LTR of M-MLV and R-MLV corroborated our preliminary conclusion that the insert of λ Xe-1 represents the right hand half of a murine leukemia virus genome. Moreover, the deletion was useful for the characterization of ligation products (Fig. 2, lane b).

In a previous study the insert of λ MLV$_{int}$ 1 was ligated to another cloned DNA fragment representing the remainder of the M-MLV genome (9). Upon transfection of mouse cells the ligation products yielded viable M-MLV. Because of the apparent resemblance between the right hand half of M-MLV and the insert of λ Xe-1 we decided to generate a hybrid virus by carrying out the aforementioned ligation experiment.

Transfection with mink lung cells but not with mouse cells resulted in the propagation of the anticipated virus XH-19. Its host range, the serological properties and electrophoretic mobilities of its gene products, the kinetics of the processing of its precursor polypeptides, and finally the physical map of its integrated provirus confirmed that XH-19 is of M-MLV origin up to the *Hin*dIII site close to the junction of the *pol* and the *env* genes. The right hand half of the new genome contains an *env* gene that is similar to two recently cloned NIH 3T3 endogenous viral sequences [14.9 and 8.13 (see above)]. This *env* gene gives the virus its xenotropic properties. Therefore it seems likely that NIH-3T3 endogenous viral sequences contain at least a complete functional *env* gene that can be expressed in a virus.

The LTRs of XH-19 deserve special attention. The presence of a 5.6 kb *Xba* I fragment in XH-19 suggests that at least part of the 1 LTRs are of M-MLV origin, because no *Xba* I site was found in the presumed 1 LTR sequence in the insert of the λ Xe-1 clone, whereas there is an *Xba* I site at 0.28 kb in the M-MLV LTR. Therefore a recombination must have occurred at this site. From a recent study (21) it is known that 1 LTRs frequently recombine.

The data reported in this paper show that a viable recombinant virus can be formed when complementary genome fragments from different viral origin are ligated. Xenotropic murine leukemia viruses play an important role in viral leukemogenesis (22), although the exact course of events has not yet become clear. All of the recombinant viruses found in tumor DNAs from leukemic mice contain xenotropic sequences that have large resemblance to the insert of the λ Xe-1 clone (23). As far as nonviral leukemia is concerned, it is worth noting that gp70 is expressed on thymoma cells of X-irradiated Swiss mice (24). A series of other new viruses constructed *in vitro* from clone λ Xe-1 could therefore be of use in a further study of leukemogenesis in mice.

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(Doctor D.R., the famous American author and connoisseur of classical music, is visiting Mr.Tortoise, one of the first acquaintances he struck up with after his immigration in Europe. They are enjoying some good wines and music.)

Mr.T.: I phoned an ancient friend of mine to join us. I'm really looking forward to introduce him to you, DeAr.

Dr.D.R.: You are always so thoughtful, Mr.T. Never will a visit to you be dull, especially not this time.

(Mr.A. hurries towards Mr.T's house as fast as is impossible even for Achillean legs.)

Mr.T.: Here you finally are, Achilles! I thought for a split second that you would not make it, just as is fortold in Zeno's paradox. Let me introduce you to Dr.DeAr, the famous computer scientist.

Mr.A.: Well, hello then. Strange name I must say. Let's shake hands to consolidate our future friendship. *(Shakes hands with D.R.)* Gee! What's this now? Something very sharp stinged my finger, blood is dripping out.

Dr.D.R.: Oh, what a terrible and bad example of my forgetfulness. It is my Voluminous RING that was broken and has a sharp edge since that day. I hope you can forgive me, Achilles, and I certainly hope you do not consider this a bad token.

Mr.A.: Don't you worry, Dr.D.R. Not yet was such a famous Greek struck down by some loss of blood.

Mr.T.: I hope you will join us in our tasting of these extremely old Bourgondies, Mr.A. Maybe also as a reinforcement?

Mr.A.: Yes, I would love to. By the way, what is that most Voluminous Music you are listening to?

Mr.T.: It is Wagner's RING, of which no Transcription was ever made Achilles. But let Dr.DeAr proceed in telling us something about his first few weeks in Europe.

Dr.D.R.: Well, as you know, I'm a foreigner here, Achilles, so as soon as I set foot on your land I joined the pCS-1, which stands for the original Circle of Strangers. Really very nice people, that make every effort to let you feel at home.

Mr.T.: Do they organize any activities that can effectively meet your

*In the spirit of D.R.Hofstadter, who composed his dialogues in the spirit of Lewis Carroll.

intellectual needs, something I regard as almost impossible, DeAr? You want some more Romanée-Conti, DeAr?

Dr.D.R.:Yes, I would like that very much. Actually some interesting wine-tastings were held in order to get rid of that most cheap Californian taste. Alas! I tend to fall asleep when drinking too much. pCS-1 also organizes television lectures, radio activities and even theatre performances to introduce us to your culture and history. Let me tell you about the last, very disturbing and fascinating, TRANSLATION. *(He empties his glass of Romanée-Conti and proceeds.)* It had the form of a Dialogue between two of the Leaders of pCS-1 and was entitled "Faction or Fiction". It was mainly a theoretical argument about the development of the democratic society between Mr.Trans and Mr.Anarchist, each representing a complementary base. I can only give some of it and my feelings about that. The strange part went like this:

Mr.A.:I would say, no statement by Faction can be proved, because no consistent system is available for that, as Gödel proved.

Mr.T.:Don´t you forget that Faction was not imposed on man. It evolved from a system, which must therefore be consistent.Its main base is: Restrictions and Ego R One.

Mr.A.:Let me, however, demonstrate before your eyes the power of Fiction by means of hypnosis. I will turn the last and most rational of our strangers here, Dr.D.R., into a Vehicle of Fiction.

(Mr.Anarchist comes over to Dr.D.R. and starts hypnotizing him. Dr.D.R. closes his eyes and dozes off. Everything gets BLURred and D.R. has a strange and disturbing dream about a visit to two completely unknown people, Mr.Achilles and Mr.Tortoise. He finds himself in Mr.T.'s house, enjoying a reverse transcript of a theme from the Musical Offering by J.S.Bach, when suddenly Mr.A. comes rushing in. When they shake hands, he feels a sharp sting in his hand.)

Mr.A.:You must apologize me, Dr.D.R., I really should have thrown this broken RING a long time ago. Please take it, Dr.D.R., maybe you can fix it some time.

Dr.D.R.:Well, thank you. I was just telling Mr.T. about my first few weeks here, Achilles, and the club I joined to get to know some people. I remember many nice activities and performances, one of which was about Anarchism in which a mysterious hypnotizing act was done. Gee! I get the strange feeling that I know this story before even having gone through it! Maybe, it is one of these Short Term Repeats the history is full of. I don´t like this at all!

(Everything turns black before his eyes and he faints. But soon a strong clapping noise pops him up.)

(The performance had apparently ended and Mr.Anarchist and Mr. Trans enjoy a standing ovation. Strangely, Dr.D.R.'s hand hurts and is quite swollen. On his finger he finds a broken RING, piercing his finger.)

(Then Dr.D.R. hears a strong clapping noise, he pops up and finds Mr.Achilles and Mr.Tortoise trying to wake him up, clapping their hands. Apparently the wine was a bit too heavy and again had made him fall asleep in the middle of his story.)

Mr.A.:It is too bad you fell asleep, DeAr, you certainly are a mysterious stranger and some day I really would like to hear the rest of what Mr.Anarchist had to say. Already I can feel some of his ideas flow through my blood like:

A virus invading my genes
Or a viper invading my jeans, sorry about that!

Dr.D.R.:It will be an honour to tell you more about the TRANSLATION, Mr.A. Then I will show you the certificate of the membership of the Circle of Mysteriously Operating Strangers that I received, bit occult though!

Mr.T.:I must say, Mr.Anarchist´s ideas and methods are kind of invasive. If not stopped by Reason, everyone in that Circle of M.O.S. will soon be one of its Vehicles of M.O.S. Indeed a disturbing thought!

Mr.A.:You have to excuse me, Mr.T., I don´t feel well at all.

Dr.D.R.:Before you leave Achilles, you must as a gift accept this RING that so unfortunately hurt your hand. Gee! Mr.A.! You should have your hand checked at the Emergency, because, forgive an American the expression, the wound is baseball-size already!

Mr.T.:I know it is unforgivable, but even under these uncomfortable and deplorable circumstances my Logic recognizes some Strange LOOPs here, recursive ones moreover. That RING is a circular object, causing accidents, while moving around in recursively, circular ways and something unknown, but related to that TRANSLATION, in the Circle of M.O.S. is the activating agent.

Dr.D.R.:I´m most honoured to notice that you read my humble book (1) so thoroughly, Mr.T. Indeed form and content of the RING seem to be identical. It generates Vehicles of M.O.S. out of members of the Circle of M.O.S.

Mr.T.:If there does exist an isomorphism between this RING and a Gödelian G String, the implication would be that the RING will blow up its Vehicle if correctly TRANSLATED, because of self-reference. Don´t you think so too, Achilles? Hey! Where did our Greek Hero, never struck down by some shedding of blood, go to?

Dr.D.R.:I think he really felt very, very sick and probably he hurried towards the bathroom. How terrible of us not to help him!

(Dr.D.R. and Mr.T. run to the bathroom upstairs, but, halfway the staircase, are halted by an enormous explosion.)

(1) D.R.Hofstadter, "Gödel, Escher, Bach: an Eternal Golden Braid". Vintage Books, New York, 1980.

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Biochemistry

Identification and molecular cloning of Moloney mouse sarcoma virus-specific sequences from uninfected mouse cells

(recombinant DNA/restriction endonuclease and S1 mapping/electron microscopy)

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ABSTRACT **When uninfected mouse cell DNA is cleaved with restriction endonuclease *Eco*RI a DNA fragment of 14 0 kilobases can be identified by hybridization to cloned DNA containing sarcoma specific sequences of Moloney mouse sarcoma virus (M MSV$_{src}$) The cellular DNA fragment contains the entire M MSV$_{src}$ specific sequences The 14 0 kilobase *Eco*RI DNA fragment was cloned in bacteriophage λ The sequence organization of a recombinant clone, λ·M$_{TV1}$ was analyzed by restriction endonuclease mapping nuclease S1 mapping and electron microscopy The results indicate that λ·M$_{TV1}$ contains an uninterrupted stretch of 1 0 kilobase similar to that found in the M MSV genome**

Murine sarcoma viruses can induce cellular transformation in cultured fibroblasts and fibrosarcomas in animals (1) Moloney murine sarcoma virus (M MSV) was isolated by passage of Moloney murine leukemia virus (M MLV) through BALB/c mice (2) The genome of a M MSV (clone 124) has been extensively analyzed and appears to have been derived by recombination between M MLV and cellular genes (3 4) Hybridization studies have shown the presence of MSV specific nucleotide sequences in the cellular DNA obtained from uninfected mouse cells (5) Recently we have cloned unintegrated M MSV circular DNA in bacteriophage λ (6) A DNA fragment encompassing the sarcoma (*src*) specific region of the M MSV genome was further subcloned in bacterial plasmid pBR322 The plasmid containing the M MSV$_{src}$ fragment showed no hybridization to the helper M MLV genomic RNA When uninfected mouse cell DNA was cleaved with restriction endonuclease *Eco*RI and analyzed on Southern blotting gels (7) by hybridization to cloned M MSV$_{src}$ probe a band migrating with a size of 14 0 kilobases (kb) can be detected The 14 0 kb band contained the entire M MSV$_{src}$ specific sequences We have cloned the 14 0 kb *Eco*RI fragment in bacteriophage λ and characterized it by restriction endonuclease mapping electron microscopy and nuclease S1 mapping The results indicate that the 14 0-kb *Eco*RI fragment contains an uninterrupted stretch of about 1000 nucleotides that is homologous to the *src* specific region of the M MSV genome

MATERIALS AND METHODS

Preparation of High Molecular Weight Cellular DNA. The procedure described by Van der Putten *et al* (8) was used Briefly cells from confluent dishes (150 mm in diameter) were scraped after brief treatment with EDTA The suspension was centrifuged at 2000 rpm for 2 min at 4 C and the pellet was resuspended in 20 vol of cold Tris/saline (10 mM Tris-HCl pH 7 5 75 mM NaCl) followed by another centrifugation at 2000 rpm for 2 min The pellet was resuspended in 10 vol of chilled lysis buffer (0 15 M NaCl 10 mM Tris HCl pH 7 5/1 mM $MgCl_2$/0 5% Nonidet P 40) and mixed well Nuclei were then pelleted by centrifugation for 2 min at 2000 rpm and the pellet was resuspended in 20 vol of saline EDTA (75 mM NaCl 25 mM disodium EDTA) To the nuclear suspension were added $NaDodSO_4$ (final concentration of 0 5%) and proteinase K (200 μg/ml) and the mixture was incubated at 37 C for 5 hr The nucleic acids were extracted with phenol chloroform and high molecular weight DNA was spooled on a glass rod after addition of chilled isopropanol The DNA was resuspended in 10 mM Tris-HCl pH 7 5 1 mM EDTA

Cloning *Subcloning of M MSV$_{src}$ specific fragment* Unintegrated M MSV circular viral DNA was cloned in bacteriophage λ and subsequently subcloned in plasmid pBR322 (6) The plasmid (p MSV 1) containing the entire M MSV genome and an additional copy of the long terminal redundancy was cleaved with restriction endonucleases *Hin*dIII and *Xba* I and fractionated on agarose gels The *Hin*dIII *Xba* I fragment was excised DNA was extracted and a stretch of 20 30 dC residues was incorporated by using terminal transferase The plasmid pBR322 was cleaved with restriction endonuclease *Pst* I and a stretch of 8 20 dG residues was added as described (9) The dC tailed insert and dG tailed plasmid were annealed and used to transform *Escherichia coli* C 600SF as described (9) The colonies were screened for drug resistance and ampicillin sensitive tetracycline resistant (Amp^{S}Tet^R) colonies were further screened by hybridization to M MSV cDNA as described (10) A recombinant plasmid (p·MSV 31) containing the *Hin*dIII *Xba* I fragment in the 3′→5′ direction clockwise to the *Eco*RI site of the plasmid was used in the studies reported here

*Cloning of 14 0 kb Eco*RI *cellular DNA* The 14 0-kb *Eco*RI fragment was excised from 0 7% agarose gel and DNA was eluted by electrophoresis as described (6) About 1 0 μg of cellular DNA was joined to 3 0 μg of isolated arms of *Eco*RI-cleaved λ Charon 4A DNA with T4 DNA ligase (11) The ligated cellular DNA and vector DNA were then packaged *in vitro* into infectious λ particles by the protocol of Blattner *et al* (11) About 9000 recombinant clones were screened with

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Abbreviations M MSV Moloney murine sarcoma virus M MLV Moloney murine leukemia virus kb kilobase(s)

labeled p MSV 31 as the probe. Plaques that hybridized to the probe were further screened and recombinant phages of interest were propagated as described (6).

All experiments were performed in accordance with National Institute of Health guidelines in P2 laboratory facilities with certified Fk2 or EK1CV vectors.

Restriction Endonuclease and S1 Analysis. The nature of the cloned DNAs was examined by restriction endonucleases as described (6). Most restriction endonucleases were obtained commercially. The restriction endonuclease digested DNA was fractionated on agarose gels ranging from 0.5 to 1.2% in 40 mM Trizma base/1 mM EDTA/5 mM sodium acetate at pH 7.8. The fragments were identified either by ethidium bromide staining (12) or by hybridization by the Southern blotting technique (7). The sizes of the restriction fragments were computed from the standard molecular weight marker in the same gel.

Endonuclease S1 mapping was performed as described by Berk and Sharp (13). The DNA and the 30S M-MSV genomic RNA were precipitated and resuspended in 10 mM EDTA. The RNA was boiled for 45 sec and kept at 4°C. The DNA preparation was boiled for 1.5 min and transferred to a 68°C water bath. a buffer containing 0.4 M NaCl, 40 mM 1,4 piperazine-diethanesulfonic acid (Pipes) (pH 6.4), 1 mM EDTA and formamide (final concentration 80%) was added. The sample was further incubated at 68°C for 15 min. The RNA was added to the DNA preparation and hybridization was carried out at 56°C for 3 hr. One hundred fifty microliters of S1 buffer containing 0.25 M NaCl, 30 mM Na acetate, 1 mM $ZnSO_4$, 20 μg of denatured calf thymus DNA per ml and 100 units of endonuclease S1 (BRL) was added to the samples. The samples were incubated at 37°C for 30 min and nucleic acids were precipitated by the addition of ethanol. The precipitate was dissolved in 10 mM Tris·HCl, pH 7.4/1 mM EDTA, pH 7.4 and analyzed on alkaline agarose gel (14). The DNA from the gel was transferred to nitrocellulose filters and the fragment was identified by hybridization to ^{32}P labeled nick translated (15) p MSV 1 DNA followed by autoradiography.

Electron Microscopy. Samples containing about 1 μg of DNA per ml and 3 μg of RNA per ml were dissolved in 80% (vol/vol) formamide, 0.4 M NaCl, 10 mM Pipes, pH 6.3, 1 mM EDTA. The DNA was heated separately at 68°C for 5 min and then mixed with RNA. The samples were incubated at temperatures that slowly decreased from 55°C to 51°C over a 4-hr period. Molecules were prepared for electron microscopy as described (16).

RESULTS

Identification of M-MSV$_{rc}$ Specific Sequences in Uninfected Cells. Fig. 1*A* shows the physical map of cloned unintegrated M-MSV DNA (p MSV 1) with *Hin*dIII cleavage site at 0 map unit. The M-MSV$_r$ specific fragment extending from *Hin*dIII to *Xba* I was subcloned in the *Pst* I site of plasmid pBR322 by the dC·dG tailing method (p MSV 31) (Fig. 1*B*). High molecular weight DNA from uninfected NIH 3T3 cells was digested with *Eco*RI and electrophoresed on agarose gels. The DNA from the agarose gel was transferred onto nitrocellulose filters and hybridized to labeled p MSV 31 DNA. A single band of a size corresponding to 14.0 kb (Fig. 1*C*) can be seen. In order to determine if the 14.0-kb cellular DNA fragment contains the entire M-MSV$_{rc}$ region we hybridized the *Eco*RI digested mouse cell DNA to *Pst* I cut DNA fragments obtained from p MSV 31. Fig. 1*B* indicates that p MSV 31 cleaved with *Pst* I generates four M-MSV$_{rc}$ specific fragments. All four viral DNA fragments hybridize to the 14.0-kb DNA fragment suggesting that the cellular DNA fragment contains a majority of the M-MSV$_r$ specific region (data not shown).

Analysis of λ·M$_{TV}$ 1 DNA. The 14.0 kb *Eco*RI fragment was cloned in bacteriophage λ Charon 4A and recombinants were screened by hybridization with p MSV 31 DNA. We obtained three reactive recombinants out of a total of 9000. Fig. 2*A* shows the *Eco*RI digestion pattern of a recombinant clone (λ M$_{TV}$ 1). The DNA was transferred to a nitrocellulose paper and hybridized to p MSV 31 DNA. Only one band migrating at a size of 14.0 kb can be detected. Fig. 2*B* shows the restriction endonuclease analysis of the λ M$_{TV}$ 1 DNA and its comparison with cloned unintegrated mouse sarcoma virus plasmid DNA (p MSV 1) (Fig. 1*A*). We chose the restriction endonucleases

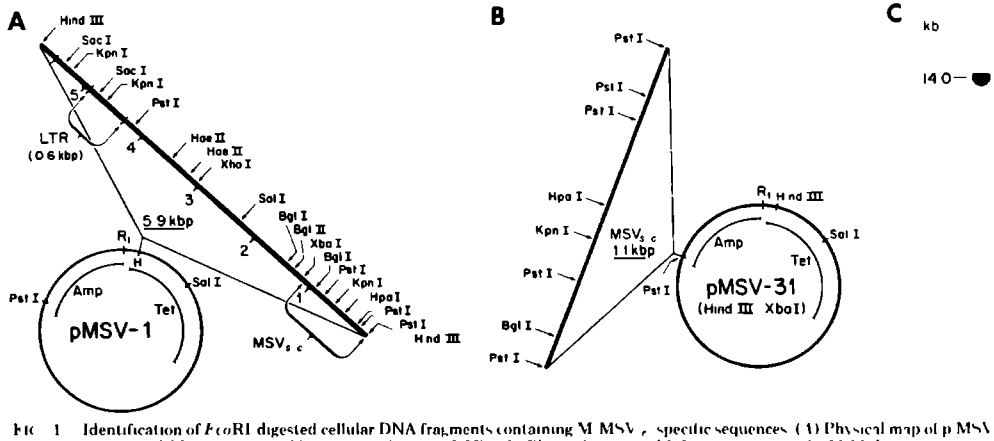

FIG. 1. Identification of *Eco*RI digested cellular DNA fragments containing M-MSV$_r$ specific sequences. (*A*) Physical map of p MSV 1 containing the entire M-MSV genome and long terminal repeat (LTR). (*B*) Physical map of p MSV 31 containing the M-MSV$_{rc}$ specific region. p MSV 1 was cleaved with *Hin*dIII and *Xba* I and subcloned in the *Pst* I site of pBR322 by dC·dG tailing. (*C*) Southern blot hybridization of *Eco*RI digested uninfected mouse cell DNA with nick translated p MSV 31 DNA. The DNA was analyzed on 0.7% agarose gel and electrophoresis was carried out for 19 hr at 1 V/cm. Hybridizations were performed at 42°C for 8 hr in 50% formamide, 0.75 M NaCl/0.075 M sodium citrate and dextran sulfate as described (17). kbp, kilo base pairs.

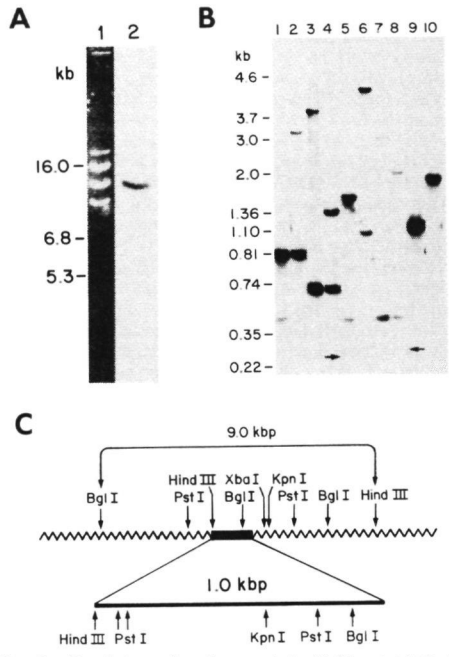

FIG. 2. Restriction endonuclease analysis of λ-M$_{TX}$-1. (*A*) *Eco*RI digestion of λ-M$_{TX}$-1. Samples were digested with *Eco*RI and analyzed on 0.7% agarose gels. Lane 1, ethidium bromide staining; lane 2; hybridization to nick-translated p-MSV-31 after Southern blot transfer. (*B*) Digestion of p-MSV-1 and λ-M$_{TX}$-1 with various restriction endonucleases followed by electrophoresis on 1.2% agarose gel. The DNA was transferred to a nitrocellulose filter and hybridized to labeled p-MSV-31 DNA. *Hin*dIII/*Bgl* I-digested p-MSV-1 (lane 1) and λ-M$_{TX}$-1 (lane 2); *Kpn* I/*Hin*dIII-digested p-MSV-1 (lane 3) and λ-M$_{TX}$-1 (lane 4); *Bgl* I/*Kpn* I-digested p-MSV-1 (lane 5) and λ-M$_{TX}$-1 (lane 6) (arrow indicates the position of the common fragment); *Pst* I-cleaved p-MSV-1 (lane 7) and λ-M$_{TX}$-1 (lane 8) (arrow indicates the position of the common small fragment); *Hin*dIII/*Xba* I-digested p-MSV-1 (lane 9) and λ-M$_{TX}$-1 (lane 10). (*C*) Tentative restriction endonuclease-generated physical map of λ-M$_{TX}$-1. kbp, Kilobase pair.

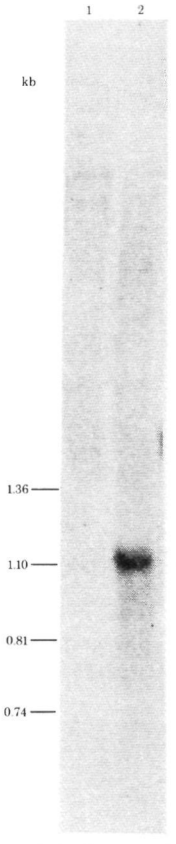

FIG. 3. S1 mapping analysis of λ-M$_{TS}$-1. Hybrids formed between λ-M$_{TX}$-1 DNA and 30S M-MSV 124 genomic RNA were digested with endonuclease S1 and analyzed on alkaline agarose gel. Lane 1, λ-M$_{TX}$-1 DNA; lane 2, λ-M$_{TX}$-1 DNA and 30S genomic M-MSV 124 RNA.

that cleave in the M-MSV$_{src}$ region for analysis. *Bgl* I and *Hin*dIII cleave p-MSV-1 to yield a fragment of about 0.9 kb and a smaller fragment of about 0.4 kb that will hybridize to p-MSV-31 DNA. Fig. 2*B* shows that *Bgl* I and *Hin*dIII digestion of p-MSV-1 (lane 1) and λ-M$_{TX}$-1 (lane 2) gives a band at about 0.85 kb in both cases. The smaller 0.4-kb fragment can be seen only in p-MSV-1 and not in λ-M$_{TX}$-1, suggesting that the sequences between p-MSV-1 and λ-M$_{TX}$-1 diverge between the two *Bgl* I sites. The larger band of about 3.0 kb in lane 2 represents the cellular DNA fragment containing a portion of M-MSV$_{src}$ specific sequences. Digestion with *Kpn* I and *Hin*dIII also gives a band of 0.7 kb in both p-MSV-1 (lane 3) and λ-M$_{TX}$-1 (lane 4). The larger band in λ-M$_{TX}$-1 of about 1.36 kb represents a DNA fragment that contains the M-MSV$_{src}$ specific region of λ-M$_{TX}$-1 and cellular sequences. The *Bgl* I/*Kpn* I digestion also gives an identical band of an average size of 0.3 kb in both p-MSV-1 and λ-M$_{TX}$-1 (lanes 5 and 6, shown with an arrow). Similarly, digestion with *Pst* I gives the smaller fragments in both p-MSV-1 and λ-M$_{TX}$-1 of identical sizes (lanes 7 and 8, shown with an arrow). [These small bands (in lanes 5–8) are very faint on the original autoradiographs and do not appear in the photographs.] When p-MSV-1 and λ-M$_{TX}$-1 are cleaved with *Hin*dIII and *Xba* I, no common fragment is observed (lanes 9 and 10). Similarly, no common fragments were observed when p-MSV-1 and λ-M$_{TX}$-1 were cleaved with *Bgl* II (data not shown). Thus, it appears that the M-MSV$_{src}$ specific sequences between p-MSV-1 and λ-M$_{TX}$-1 diverge between *Bgl* I and *Xba* I sites. A tentative map of the λ-M$_{TX}$-1 DNA is shown in Fig. 2*C*.

Organization of M-MSV$_{src}$ Specific Sequences in λ-M$_{TX}$-1. Based upon the restriction endonuclease data (Fig. 2), the M-MSV$_{src}$ sequences in λ-M$_{TX}$-1 appear to have the same organization as seen in the M-MSV genomic RNA (Fig. 1*A*). These results, however, do not show whether the *src*-specific sequences present in λ-M$_{TX}$-1 are present as a continuous stretch or have some intervening sequences. In order to approach this question, we did the following two kinds of experiments:

(*i*) *S1 mapping.* λ-M$_{TX}$-1 DNA was hybridized to M-MSV genomic RNA under conditions of R-loop formation. The hybrid was treated with single-strand-specific nuclease S1 and

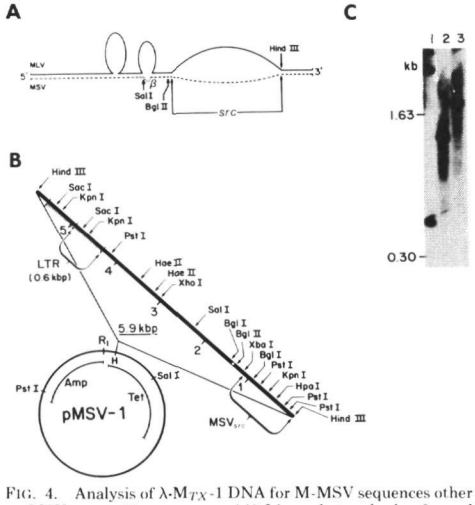

FIG. 4. Analysis of λ·M$_{TX}$-1 DNA for M-MSV sequences other than MSV$_{src}$ specific sequences. (*A*) Linear heteroduplex formed between M-MLV cDNA and M-MSV 30S genomic RNA (4). β denotes the small substitution loop. (*B*) Physical map of p·MSV-1. (*C*) p·MSV-1 DNA cleaved with *Pst* I (lane 1), *Bgl* II and *Hin*dIII (lane 2), and *Sal* I and *Bgl* II (lane 3), followed by Southern blotting transfer and hybridization to labeled λ·M$_{TX}$-1 DNA.

analyzed on alkaline agarose gels. The DNA from the gel was transferred to nitrocellulose filters and hybridized to nick-translated p·MSV-1 DNA representing the entire genome. Fig. 3 (lane 1) shows that the λ·M$_{TX}$-1 DNA that was not annealed to M-MSV RNA shows no distinct band, whereas after annealing to M-MSV genomic RNA a 1.0-kb DNA fragment can be seen (lane 2). The detection of a single DNA fragment of an average size of 1.0 kb suggests that the λ·M$_{TX}$-1 DNA contains the M-MSV$_{src}$ specific sequence as a continuous stretch.

(*ii*) *Hybridization with λ·M$_{TX}$-1 DNA.* We wanted to determine if λ·M$_{TX}$-1 DNA contains short M-MSV sequences that are not continuous with M-SMV$_{src}$ specific sequences and escaped detection by S1 mapping. Of particular interest was the small substitution loop β seen in Fig. 4*A*. The total λ·M$_{TX}$-1 DNA was labeled by nick translation and hybridized to various restriction fragments obtained from p·MSV-1. Fig. 4*B* shows that p·MSV-1 cleaved with *Pst* I (lane 1), *Bgl* II and *Hin*dIII (lane 2), and *Bgl* II and *Sal* I (lane 3) should yield a fragment of about 1.0 kb containing sequences present in the small substitution loop, β. The *Bgl* II/*Hin*dIII fragment contains the majority of M-MSV$_{src}$ specific sequences and should hybridize to λ·M$_{TX}$-1 DNA. The results in Fig. 4*C* show that the *Bgl* II/*Hin*dIII fragment of an average size of 1.2 kb can be detected, whereas no *Bgl* II/*Sal* I fragment of an average size of 1.0 kb can be seen. The *Bgl* II/*Sal* I fragment of an average size of 1.7 kb can be seen, which represents the *Bgl* II to *Hin*dIII region of the plasmid and *Hin*dIII to *Sal* I fragment (0.65 kb) of the plasmid, pBR322. Thus, it appears that only sequences corresponding to M-MSV genome present in the λ·M$_{TX}$-1 DNA are an uninterrupted stretch of about 1.0 kb.

Characterization of λ·M$_{TX}$-1 DNA by Electron Microscopy. It is possible that there are several copies of M-MSV$_{src}$ specific sequences in the 14.0-kb fragment. However, when λ·M$_{TX}$-1 was heteroduplexed to MSV genomic RNA, only a

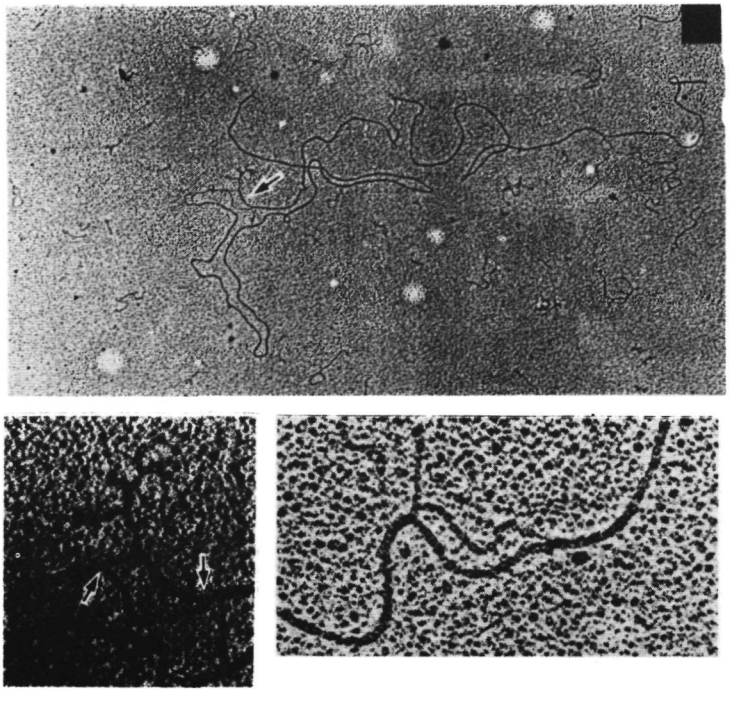

FIG. 5. Electron micrograph of the R loop formed between λ·M$_{TX}$-1 DNA and 30S M-MSV genomic RNA. (*Upper*) λ·M$_{TX}$-1 DNA was annealed to M-MSV genomic RNA and analyzed as described (16). The arrow indicates the R loop formed. (*Lower*) Enlargement of the area showing the R loop. Two different molecules are shown.

single R loop structure of an average size of 1.0 kb was observed (Fig. 5 *Upper*). The region showing the R loop structure in Fig. 5 *Upper* (marked with an arrow) is shown in Fig. 5 *Lower*. Fig. 5 *Lower* also shows the region of R loop structure from another molecule. The unhybridized RNA appears as a condensed structure with a shorter end (presumably the 3' end) and a longer stretch (presumably the 5' end) at either end of the R loop. Thus it appears that λ-MTλ 1 DNA contains only one copy of the M-MSV$_{src}$ specific sequences.

DISCUSSION

Mouse cell DNA contains sequences homologous to the MSV$_{src}$ specific region. By use of M-MSV$_{vr}$ specific DNA as a probe, a single *Eco*RI digested mouse cell DNA fragment of 14.0 kb can be identified. The 14.0-kb fragment showed no detectable hybridization to M-MLV cDNA, suggesting that it contains no viral sequences other than the M-MSV$_{src}$ specific sequences. Occasionally we have also observed a 5.3 kb fragment that hybridized to M-MSV$_{src}$ probe. This fragment contained the entire M-MSV$_{src}$ specific sequences. The origin of this fragment remains obscure. The 14.0 kb fragment contains a single copy of M-MSV$_{src}$ specific sequences (Fig. 5). A 14.0 kb *Eco*RI fragment in uninfected mouse cells has also been detected by Vande Woude *et al.* (18) and Tronick *et al.* (19).

The recombinant clone λ MTλ 1 contained a continuous stretch of 1.0 kb that was homologous to M-MSV genomic RNA. This size corresponded to the M-MSV$_{src}$ specific sequences in the M-MSV genome. The λ-MTλ 1 DNA was cleaved with several restriction endonucleases that also cleave the M-MSV$_{src}$ specific region. The *Bgl* I *Hin*dIII restriction fragment (Figs. 1 and 2) is present in the λ-MTλ 1 DNA. Furthermore analysis of λ-MTλ 1 DNA with *Kpn* I *Pst* I (Fig. 2) and *Hpa* I (data not shown) also generate the same fragments as observed for the plasmid DNA containing the entire viral genomic sequences. *Xba* I cleaves p-MSV 1 DNA and is located close to the *Bgl* I site (Fig. 2, lanes 9 and 10). Thus it appears that endogenous transforming gene sequences in the M-MSV$_{src}$ specific region extend between *Bgl* I and *Hin*dIII restriction sites and diverge between *Bgl* I and *Xba* I sites.

The S1 and restriction endonuclease mapping data suggest that the cellular sequences involved in the biogenesis of M-MSV are completely conserved in the viral genomic RNA. Earlier hybridization studies done with *src* specific probes had suggested a large degree of homology between viral *src* specific and cellular DNA sequences (5). The cellular sequences containing M-MSV$_{vr}$ specific sequences alone do not appear to be able to transform cells (M. H. T. Lai and I. M. Verma, unpublished data). They apparently need to be inserted into the viral genome to become a transforming gene. The viral sequences upstream may serve as promoter for transcription of MSV$_{vr}$ specific RNA. We have identified a 21S subgenomic mRNA species from M-MSV infected cells that directs the synthesis of a 37,000-dalton protein *in vitro* (unpublished results). *In vitro* translation of viral genomic RNA also directs the synthesis of a 37,000-dalton protein in addition to other viral proteins (20). The synthesis of the 37,000 dalton protein is selectively inhibited if the viral RNA is preannealed to p-MSV 31 DNA (unpublished results). The 37,000 dalton protein will require a coding capacity of approximately 1100 nucleotides. The cellular DNA sequences containing M-MSV$_{src}$ sequences are about 1.0 kb.

Note Added in Proof. We have determined the complete nucleotide sequence of the M-MSV$_{src}$ gene and portions of M-MSV$_{vr}$ specific sequences in λ MTλ 1. Based upon the nucleotide sequence data, the *Pst* I site between *Kpn* I and *Bgl* I (Figs. 1*A* and *B* and 4A) should be placed between *Bgl* I and *Xba* I sites (Figs. 1*A* and 4A).

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CHAPTER VI

Molecularly Cloned c-mos(rat) is Biologically Active

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ABSTRACT

A unique rat cellar gene, c-mos(rat), homologous to the transforming sequences, v-mos, of Moloney murine sarcoma virus (M-MSV) was detected by hybridization to a v-mos specific probe and molecularly cloned together with its flanking sequences in an 11 kbp EcoRI DNA fragment inserted in vector Charon 4A. Two probes were used to investigate the position and orientation of c-mos(rat) in the clone examined (D_3e), namely pMSV-31 which contains the sequences specific for the transforming sequences of M-MSV and pCS-1 which harbors 0.5 kbp of 5'-terminal sequences of c-mos(mouse) as well as 0.7 kbp of its flanking sequences. After ligation of a restriction fragment of D_3e containing c-mos(rat) to a fragment containing the long terminal repeat (LTR) of M-MSV and transfection of the DNA onto rat cells, we detected foci of transformed cells, thus showing that c-mos(rat) is biologically active. Using DNA fragments derived from D_3e we studied the conservation of c-mos and of its flanking sequences in several species. c-mos(rat) as well as some of its flanking sequences appeared to be very much conserved in the species studied.

INTRODUCTION

M-MSV causes rapidly developing sarcomas in mice as well as malignant transformation of cultured fibroblasts (Moloney, 1966). The viral gene responsible for the oncogenic properties is called v-mos (v from viral). The virus acquired this gene, and indeed its very identity, by a recombination between Moloney murine leukemia virus (M-MLV) and mouse cellular sequences, designated c-mos (c from cellular) (Van Beveren et al., 1981[a]). The mos gene belongs to a group of about 13 originally cellular genes, collectively called oncogenes, that through recombination events gave rise to about 20 new transforming type C RNA tumor viruses (Coffin et al., 1981). Much research is focussed on cellular homologues of these genes, because it is believed that their abnormal expression may be involved in non-viral tumorigenesis. In fact, recent reports describe the expression of some oncogenesin human tumors or in

cell lines derived from human tumors (Der et al., 1982, Eva et al., 1982, Goldfarb et al., 1982). In addition to potentially malignant properties, the c-onc sequences are believed to fulfill, yet unknown, important biological functions, possibly in development and differentiation of the organism (Eva et al., 1982, Westin et al., 1982). This speculation is mainly based on the slow evolutionary rate of these sequences. Homologues of c-onc genes are usually found in a wide variety of vertebrates (Stéhelin et al., 1976, Frankel & Fischinger, 1977, Spector et al., 1978, Sheiness & Bishop, 1979, Goff et al., 1980, Shih & Weinberg, 1981).

Unlike some other oncogenes, the c-mos gene is not very well characterized. The physical and biological properties of the viral gene product are not well known. Only recently, very low levels of v-mos related protein could be detected in M-MSV transformed cells (Papkoff et al., 1982). The product was characterized in in vitro translation studies (Papkoff et al., 1981). In mouse (Jones et al., 1980, Oskarsson et al., 1980) and man (Watson et al., 1982) cloned c-mos sequences are described. The c-mos(mouse) gene exists in a hypermethylated form in mouse cells: no expression is observed (Gattoni et al., 1982). In this paper we report data on c-mos(rat) and its transfection into rat cells. This study led also to the discovery of a unique and very conservative sequence that flanks c-mos of mouse and rat and is present in other vertebrates.

MATERIALS AND METHODS

Cells and transfection procedure

The cell lines 3Y1 (rat), Rat-2 (rat), NIH-3T3 (mouse), Balb/c-3T3 (mouse), CCL-64 (mink), PK-15 (pig), AZ (cat) and Halec (hamster) were grown on Dulbecco's modification of Eagle's medium, supplemented with 10 % calf serum (GIBCO) and antibiotics. Transfection was performed essentially as described by Graham & Van der Eb (1973). In short: Rat-2 cells were plated 4 hr prior to transfection in petridishes (Costar, 35 mm). 20 Min after precipitation of cloned DNA fragments with $CaCl_2$ the DNA precipitate was applied to the cells and incubated for 5 hr. After removal of the medium, the cultures were incubated for one hr after which they were transferred to 100 mm petridishes (Costar). Cells were fed medium every three days and foci were counted after 2-3 weeks.

Cloning procedure

Rat cellular DNA was isolated from 3Y1 cells as described (Van der Putten et al., 1979) and 100 µg DNA were digested with EcoRI (Boehringer) and sepa-

rated on a 0.7 % agarose gel (SeaKem). The gel was sliced and DNA was electro-eluted (Allington et al., 1978). Fractions containing sequences homologous to v-mos were detected after blotting on nitrocellulose filters by the Southern technique (Southern, 1975) by hybridization to pMSV-31. Hybridizations were performed at 42° C for 15 hr in 50 % formamide, 5xSSC, 1xDenhardt's solution, 0.02 M sodium phosphate, 100 µg/ml denatured herring sperm DNA and 0.5-1x10^6 cpm/ml of probe DNA. The final wash of the filters was for 15 min at 62° C in 0.1xSSC/0.1 % SDS (Jones et al., 1980). An 11 kbp fragment containing c-mos homologous sequences was cloned in Charon 4A as described (Jones et al., 1980). From 100,000 plaques screened we obtained 15 plaques harboring phages with v-mos homologous sequences. Five of these were grown and appeared to contain the same insert. One of these, D_3e, was used for characterization.

Clones used

pMSV-31: pBR322 containing the v-mos sequences as insert in the PstI site (Jones et al., 1980). pCS-1: pBR322 containing 0.5 kbp of 5'-terminal c-mos (mouse) sequences as well as 0.7 kbp of its flanking sequences (Van Beveren et al., 1981[b]). pMLTR: pBR322 containing two adjacent LTRs and flanking sequences from M-MSV, inserted in the EcoRI and PstI sites of the plasmid.

Restriction enzyme analysis and ligation of DNA fragments

DNAs were digested with EcoRI (Boehringer), KpnI, PstI, XbaI, BamHI, SmaI or HindIII (Bethesda Research Labs) as prescribed by the manufacturers. After separation on 0.7 % agarose gels the DNA fragments were processed as described under " cloning ". DNA fragments to be ligated were isolated from gel slices by electro-elution, ethanol precipitated twice and ligated using T4 DNA ligase (Boehringer).

RESULTS

Characterization of c-mos(rat)

In order to detect rat cellular sequences, homologous to v-mos, and to compare them to c-mos(mouse), we isolated high molecular weight DNA from 3Y1 rat cells and NIH-3T3 cells. DNAs were digested with EcoRI, PstI or with a combination of EcoRI and HindIII, separated on an agarose gel, blotted and hybridized to nick-translated pMSV-31 or pCS-1. The results are shown in Fig.1.

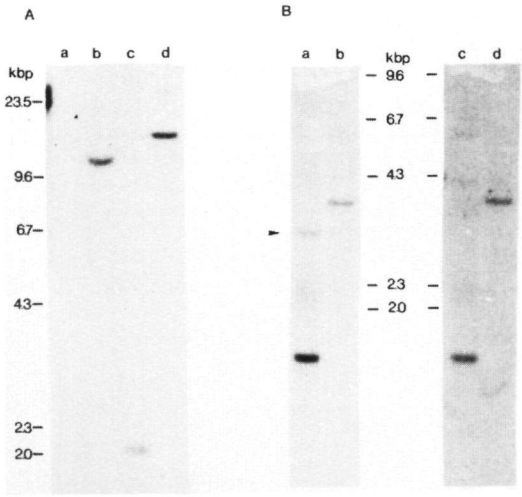

Figure 1: Detection of c-mos(rat). A) High molecular weight DNAs from rat (lanes a and b) or from mouse (lanes c and d) were digested with PstI (lanes a and c) or with EcoRI (lanes b and d). After electrophoretic separation on an agarose gel and blotting onto a nitrocellulose filter the DNA fragments were detected by hybridization to pMSV-31. B) In HindIII/EcoRI digested rat DNA (lanes a and c) or mouse DNA (lanes b and d) c-mos and flanking sequences were detected using pCS-1 (lanes a and b) or pMSV-31 (lanes c and d) as probes.

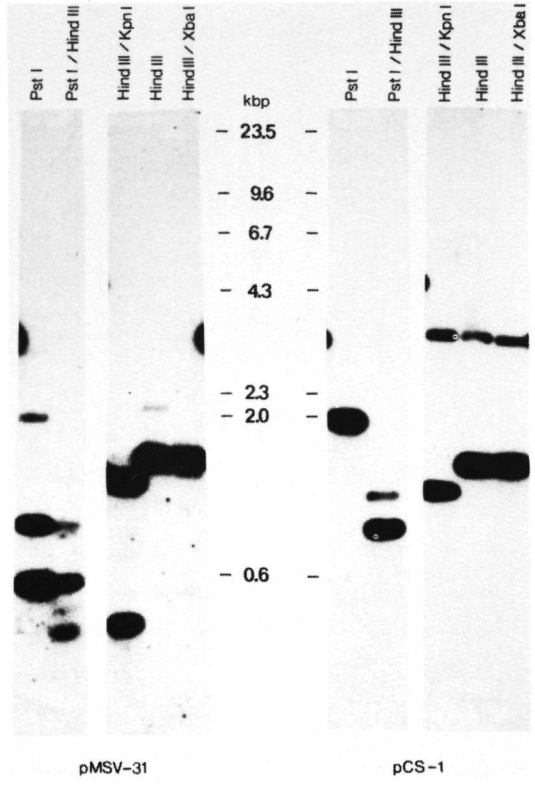

Figure 2: Positioning of c-mos (rat) in clone D_3e. 2 ng of D_3e DNA was first digested with EcoRI and next with the enzymes indicated. Detection of DNA fragments was as described in the legend to Fig.1.

From Fig.1A it can be seen that mouse DNA contains c-mos sequences hybridizing to pMSV-31 on one EcoRI fragment of 14 kbp (lane d) and on a PstI fragment of 2.0 kbp (lane c). Several small PstI DNA fragments hybridize to pMSV-31 (Jones et al., 1980), but they are not detectable in this experiment. A rat EcoRI fragment of 11 kbp hybridizes to pMSV-31 (lane b): some PstI fragments reactive with pMSV-31 as a probe were too weak to be seen in this experiment (lane a).

A further characterization was obtained using pCS-1 as a probe in a comparison between mouse and rat DNAs. In Fig.1B this comparison is shown. Lanes b and d show an EcoRI/HindIII

digestion of mouse DNA: a single fragment of 3.9 kbp hybridizes to pCS-1 (lane b) and to pMSV-31 (lane d) as was expected from earlier restriction data (Jones et al., 1980). However, EcoRI/HindIII digested rat DNA reveals one DNA fragment of 1.5 kbp hybridizing both to pCS-1 and pMSV-31 (lanes a and c) as well as an additional fragment of 3.2 kbp hybridizing only to pCS-1 (lane a), indicated by the arrow. Therefore it appears that rat DNA contains sequences homologous to v-mos as well as sequences homologous to mouse DNA juxtaposed to the c-mos(mouse) gene. It will follow from the experiments described below that in the rat genome these two sequences are also very close.

Restriction mapping of c-mos(rat)

The EcoRI fragment, harboring c-mos related sequences (see Fig.1A, lane b) was cloned as described. Five out of 15 positive clones were grown and digested with EcoRI and HindIII and analysed on agarose gels. They appeared to be indistinguishable by restriction analysis and to contain an 11 kbp EcoRI DNA insert. One of these clones, D_3e, was used for further analyses.

By using pMSV-31 and pCS-1 as probes we determined the position of the c-mos gene on the 11 kbp fragment. Fig.2 shows part of these restriction analyses. The left panel shows an autoradiograph of a blot containing D_3e restriction fragments hybridized to pMSV-31 and the right panel shows an autoradiograph of an identical blot hybridized to pCS-1. The additional DNA fragments showing up on the right panel result from hybridization of rat DNA sequences to the mouse sequences flanking c-mos(mouse). Although the environment of c-mos in mouse and rat is not completely identical (Fig.1) this experiment shows that sequences in the neighborhood of both genes are homologous. HindIII digestion results in fragments of 1.5 and 3.2 kbp. The latter fragment is only detectable using pCS-1 as a probe as was expected from the results shown in Fig.1B. This fragment is cleaved by XbaI resulting in a slightly smaller fragment of 3.1 kbp (Fig.2, right panel). KpnI cleaves the c-mos sequences once: the small KpnI/HindIII fragment of 450 bp can only be detected using pMSV-31 as a probe. In this Figure PstI fragments are observed containing c-mos(rat) sequences and flanking sequences that were not detectable in the experiment shown in Fig.1. From these and other data a restriction map of clone D_3e was obtained. In Fig.3 it is compared to the restriction map of c-mos(mouse) (Jones et al., 1980, Oskarsson et al., 1980). Also the regions in D_3e hybridizing to pMSV-31 and to pCS-1 are indicated.

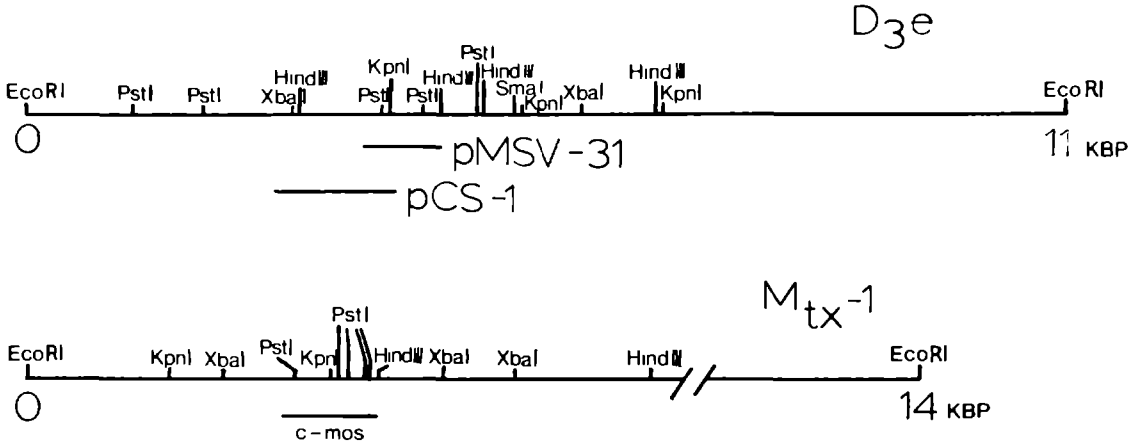

Figure 3: Restriction map of clone D_3e. The restriction map of D_3e containing c-mos(rat) is compared to the restriction map of clone M_{tx}-1 (Jones et al., 1980) containing c-mos(mouse). Fragments hybridizing to pMSV-31 or to pCS-1 are underlined.

Biological activity of c-mos(rat)

As was shown by Blair et al. (1981) the c-mos(mouse) gene has no transforming activity in transfection assays unless the gene is ligated to activating sequences like retroviral LTRs (Temin, 1982). We isolated LTR sequences from a plasmid pMLTR that contains two M-MSV LTRs in the same orientation by digestion with SmaI. Nucleic acid sequence data showed the presence of a single SmaI site in the 3´region of this LTR (Van Beveren et al., 1981[a]). The resulting 0.6 kbp fragment containing the cyclically permuted LTR sequences, was ligated to a SmaI fragment harboring c-mos(rat) (Fig.4).

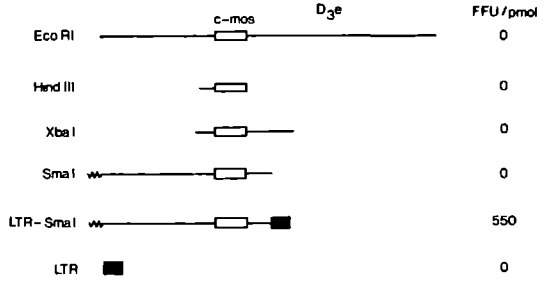

Figure 4: Biological activity of c-mos(rat). The EcoRI insert of D_3e, fragments thereof, LTR sequences or the D_3e fragment ligated to the LTR sequences were transfected onto rat cells and transformed colonies were counted after 2-3 weeks. FFU/pmol = focus forming units per pmol DNA.

The ligated DNA as well as several other DNA fragments were transfected onto rat cells and transformed colonies were counted after 2-3 weeks. Fig.4 shows the results of the transfection experiments. It can be seen that the 11 kbp EcoRI insert or the LTR sequences are not biologically active by themselves.

Also, removal of large parts of the adjacent rat cellular sequences by digestion with HindIII or XbaI, does not result in detectable activity. However, the DNA preparation consisting of c-mos(rat) ligated to LTR sequences gives rise to transformation of cells in tissue culture after transfection. Therefore clone D_3e contains a functional c-mos(rat) gene that can be activated by ligation to activating LTR sequences.

Conservation of rat cellular sequences in other species

Several cellular homologues of viral transforming sequences have been detected in the DNA of other species than the species from which the viruses were isolated originally (Stéhelin et al., 1976, Ellis et al., 1980, Goff et al., 1980, DeFeo et al., 1981). Thus, also clone D_3e could be used to investigate the presence of cellular sequences homologous to c-mos(rat) in other species. Moreover, the fact that mouse DNA present in pCS-1, other than c-mos, hybridized to a unique fragment in rat DNA, present in clone D_3e, suggested that this DNA sequence juxtaposed to c-mos in mouse and rat might be conserved throughout several species.

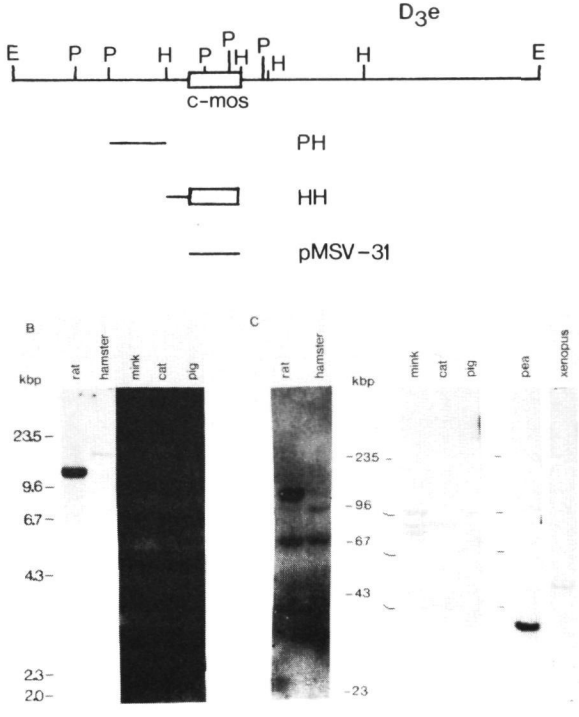

Figure 5: Conservation of c-mos and its flanking sequences. A) Map of the fragments used as probes for detection of c-mos related sequences or its flanking sequences (probe P-H). E = EcoRI, P = PstI and H = HindIII. B) Autoradiograph of a nitrocellulose filter containing EcoRI fragments of the species indicated, hybridized to pMSV-31. The exposure times were one day for the lanes marked rat and hamster and ten days for the other lanes. C) Autoradiograph of a nitrocellulose filter containing Eco RI fragments of the species indicated, hybridized to probe P-H. The exposure times were two days for the lanes marked rat and hamster and 7 days for the other lanes.

Therefore we isolated two restriction fragments, designated P-H and H-H from clone D_3e as indicated in Fig.5A. These fragments as well as pMSV-31 were nick-translated and used as probes to screen filters containing separated EcoRI fragments of hamster, rat, mink, cat, pig, xenopus and pea DNAs. From Fig.5B it can be seen that pMSV-31 recognizes sequences homologous to v-mos in various mammalian DNAs. Each of the DNAs from hamster, mink, cat and pig contain one EcoRI fragment hybridizing to pMSV-31 (respectively of 15, 4.9, 9.6 and 5.4 kbp). In xenopus and pea DNAs no fragments were detected with pMSV-31 under the hybridization conditions described (not shown). When using H-H as a probe we obtained the same results (not shown).

We then investigated whether or not similar DNA fragments could be detected with probe P-H. Fig.5C shows the results obtained with the nick-translated P-H probe. In rat DNA three bands are detected, namely the 11 kbp fragment containing c-mos, a 9.5 and a 6.7 kbp fragment. In hamster DNA two bands are visible that comigrate with the 9.5 and 6.7 kbp bands found in rat DNA. In mink DNA three bands show up; however, no 4.9 kbp band is visible with probe P-H whereas this band was detected using pMSV-31 as a probe. In cat DNA one band (8.0 kbp), in pig DNA one band (9.6 kbp) and in xenopus DNA one band (5.0 kbp) show up. A hybridizing band of 3.9 kbp is detected in pea DNA, although we never detected fragments in pea DNA using pMSV-31 as a probe. The data shown indicate that sequences homologous to c-mos and to its juxtaposing sequences are present in low copy numbers in closely or more distantly related species. All species examined contained DNA fragments homologous to rat cellular sequences represented in probe P-H, but only in mouse and rat these sequences lie next to c-mos on one EcoRI fragment.

DISCUSSION

In this report we show that sequences, homologous to Moloney murine sarcoma virus specific sequences (v-mos) are present in normal rat DNA on one unique 11 kbp EcoRI fragment. These sequences can be activated to transform cells in vitro upon transfection. Recently Watson et al. (1982) reported that the human c-mos gene could not be activated in similar transfection experiments. The detection of a long open reading frame made it unlikely that a mutation caused the absence of transforming activity. Rather it might be that c-mos(human) has diverged from c-mos(mouse) and the closely related c-mos(rat), thereby losing the capacity to transform cells. The position

of c-mos on clone D_3e was determined using pMSV-31 as a probe and the orientation was determined using pCS-1 as a probe. The orientation of the strand homologous to M-MSV RNA in clone D_3e is from the left to the right in Fig.3.

Type C retroviral LTRs or parts have been used succesfully to activate genes (Blair et al., 1981, DeFeo et al., 1981, Temin, 1982). Nucleic acid sequence data have shown that they harbor a termination signal, a poly-A addition site and promoter region. In addition, sometimes they contain repeated sequences of variable length, that can be used as enhancer sequences in experiments designed to activate genes of interest (Levinson et al., 1982). In similar experiments we used the permuted M-MSV LTR, obtained by cleaving pMLTR with SmaI. The SmaI site is located to the right of the promoter sequences in this LTR (Van Beveren et al., 1981[a]). The ligated DNA preparations, used for transfections, appeared to consist for the major part of the product shown in Fig.4, as well as of DNA consisting of concatemers containing several LTRs (not shown). For efficient transfection to occur the ligation step was necessary. The transfection efficiency we observed was much lower than the efficiency reported for c-mos(mouse) (Blair et al., 1981).

Using a cDNA, specific for the transforming gene (src) of Rous sarcoma virus (a chicken virus) Stéhelin et al. (1976) found that the degree of homology between the src gene and its cellular homologues decreased with increasing evolutionary distance between the species investigated and the chicken. Cellular homologues of transforming genes of RNA tumor viruses are present in many different animal species (Coffin et al., 1981). In our assay, using specific probes (Fig.5A), we found that the degree of homology between v-mos and c-mos was highest for mouse and rat and less for mink, hamster, cat and pig. Interestingly, probe P-H, representative for sequences juxtaposed to c-mos both in mouse and rat, detected homologous sequences in low copy number in all species examined, including a plant. However, only in the case of rat and mouse is the same EcoRI fragment detected using probes P-H and pMSV-31. The detection of different fragments with these probes for hamster, mink, cat and pig could be caused by the presence of an additional EcoRI site or by a different organization of the sequences analysed. The 3.9 kbp fragment detected in pea DNA, under rather stringent hybridization conditions, might, however, be caused by a fragment present as a repetitive sequence with some homology to probe P-H. We do not know the relevance of this sequence, but the close association of these unique sequences to c-mos in rat and mouse and the conservation throughout other species suggest that they fulfill some important function.

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Het gebied der tumorvirologie is de laatste jaren dermate uitgebreid dat de kloof tussen goed gedefinieerde experimenten aan de ene kant en een begrip van tumorontwikkeling bij de mens aan de andere kant vrij spoedig overbrugd kan worden. De kennis omtrent RNA tumorvirussen zal hier het zijne toe bijdragen.

RNA tumorvirussen (Oncovirinae) vormen een van de drie subfamilies van de Retroviridae en worden onderverdeeld op basis van morfologie in type B, type C en type D virussen. Tot de best bestudeerde virussen behoren het prototype van de type B virussen, Mouse mammary tumor virus, en enkele virussen behorend tot de type C virussen, zoals de muizeleukemievirussen (MLV) en de muizesarcomavirussen (MSV). De zoogdier type C virussen kunnen verder onderverdeeld worden op basis van wijze van transmissie, pathogeniciteit, gastheerschap, en replicatie competentie. Zo zijn MSVs replicatie defectief. Ze veroorzaken solide tumoren in dieren en transformeren cellen in weefselkweek. MLVs zijn replicatie competent en veroorzaken geen of zich langzaam ontwikkelende tumoren in dieren.

Er zijn nu ongeveer 30 verschillende transformerende virussen bekend. Zij herbergen een transformerend gen (v-onc). Op verschillende wijzen werd aangetoond, dat deze virussen ontstonden als gevolg van een of meer recombinatie gebeurtenissen tussen virale sequenties en gastheer-sequenties (c-onc). c-onc genen blijken zeer conservatief te zijn en zijn mogelijk betrokken bij het ontstaan van niet-virale tumoren bij de mens, zoals beschreven in Hoofdstuk I.

Hoofdstuk II beschrijft de interactie tussen een MLV, AKR virus, en rattecellen. AKR virus kan slechts muizecellen infecteren en werd daarom als DNA in rattecellen ingebracht via micro-injectie. Bestudering van de integratie en expressie in een gecloneerde cellijn toonde dat de rattecellen een of meer factoren missen, nodig voor juiste splitsing van een precursor eiwit van AKR virus, dat normaal rijpe envelop-eiwitten oplevert.

De kinetiek van splitsing van een ander AKR precursor-eiwit, dat normaal rijpe core eiwitten oplevert, werd bestudeerd in muize cellen, gemicro-injecteerd met een stuk AKR viraal DNA, zoals beschreven in Hoofdstuk III. Het bleek dat dit stuk DNA zeer waarschijnlijk een recombinatie heeft ondergaan met viraal DNA, dat endogeen in groten getale voorkomt in muize

chromosomaal DNA.

In Hoofdstuk IV wordt het gen beschreven, dat codeert voor het envelop precursor-eiwit van een van de endogene muizevirussen. Dit gecloneerde gen werd gekoppeld aan het resterende DNA van Moloney muizeleukemievirus. Na transfer van dit DNA in nertscellen kon een recombinant virus, XH-19, geisoleerd worden. Het XH-19 envelop gen en zijn producten werden nader gekarakteriseerd.

De Interlude tussen de hoofdstukken IV en V is een hommage aan D.R. Hofstadter. Het benadrukt enkele vreemde aspecten van v-onc en c-onc genen.

De Hoofdstukken V en VI behandelen de clonering en karakterisering van respectievelijk het muize en het ratte cellulaire homoloog van het transformerende gen (v-mos) van Moloney muizesarcomavirus. De nadruk lag hierbij op de homologie tussen v-mos en c-mos en de biologische activiteit van het rattehomoloog.

CURRICULUM VITAE

Franciscus Augustinus Maria Jozef van der Hoorn werd geboren te Heerlen op 24 augustus 1954. Na het behalen van het diploma Atheneum-B aan het Bernardinuscollege te Heerlen werd in 1972 gestart met de studie scheikunde aan de Katholieke Universiteit te Nijmegen. In februari 1978 werd het doctoraal examen behaald met als hoofdrichting Biochemie (Prof. Dr. H.Bloemendal) en als bijvakken Farmacochemie (Prof. Dr. J.M. van Rossum) en Russische Taalkunde (Prof. Dr. Z. Anthonisse, o.f.m.). Van maart 1978 tot september 1982 heeft hij als wetenschappelijk medewerker onderzoek verricht aan de expressie van retrovirale genen onder leiding van Prof. Dr. H.P.J. Bloemers op het Laboratorium voor Biochemie van de Katholieke Universiteit te Nijmegen (Hoofd: Prof. Dr. H. Bloemendal). In april 1979 werd 4 weken onderzoek verricht in de groep van Dr. T. Graf, Max Planck Institut für Virusforschung, Tübingen, ondersteund door middel van een EMBO short term fellowship. Van mei 1979 tot december 1979 was hij te gast in de groep van Dr. I. M. Verma, Tumor Virology Laboratory, The Salk Institute, San Diego teneinde zich recombinant DNA technieken eigen te maken, financieel gesteund door het Koningin Wilhelmina Fonds.

Vanaf februari 1983 zal hij werkzaam zijn op het Institut Suisse de Recherches Experimentales sur le Cancer te Epalinges s./Lausanne op het laboratorium van Dr. H. Diggelmann.

Stellingen behorend bij het proefschrift " Expression of retroviral genes after DNA transfer" door F.A.M.J. van der Hoorn

1. Indien de EcoRI restrictie enzym gegevens van de rattecellijnen NX-2 en NX-3, gepubliceerd door Steffen & Weinberg, juist zijn, hebben Yoshimura & Yamamura deze cellijnen verwisseld.

   Steffen,D. & Weinberg,R.A.(1978). Cell 15,1003.
   Yoshimura,F.K. & Yamamura,J.M.(1981). J.Virol.38,895.

2. Het ontbreken van een band met wat hoger molecuulgewicht in restrictie enzym analysen is vaak een gevolg van de kwaliteit van het DNA. Zo ontbreekt in de analysen van Pulciani et al. het 23 kbp EcoRI DNA fragment waarop c-ras(muis) gelocaliseerd is, dat door Ellis et al. eerder beschreven werd.

   Pulciani,S. et al.(1982). Proc.Natl.Acad.Sci.USA 79,2845.
   Ellis,R.W. et al.(1980). J.Virol.36,408.

3. Het ontbreken van een positief resultaat in transfectie assays is vaak een gevolg van de kwaliteit van het DNA. Zo leidt transfectie van DNA van de cellijn MCF-7, een menselijke borsttumorcellijn, in handen van Pulciani et al. niet tot transformatie van NIH-3T3 cellen, hoewel Lane et al. reeds eerder in dezelfde soort assays de transformerende sequenties van de MCF-7 cellijn geidentificeerd hebben.

   Pulciani et al.(1982). Proc.Natl.Acad.Sci.USA 79,2845.
   Lane,M.-A. et al.(1981). Proc.Natl.Acad.Sci.USA 78,5185.

4. De conclusie van Joseph, dat vorming van vorm I retroviraal DNA ook na beëindiging van celdeling doorgaat, is, gezien de magere experimentele opzet, totaal speculatief.

   Joseph,D.R.(1981). J.Virol.38,1095.

5. Tot het ontbreken van een cultivar/temperatuur interactie voor zaailingen zoals vermeld door Parlevliet, kan niet geconcludeerd worden uit tabel 2, daar de berekeningen uitgevoerd voor deze tabel niet overeenstemmen met de berekeningen vermeld in de Materialen & Methoden.

   Parlevliet,J.E.(1975). Euphytica 24,21.

6. Gezien het gemak waarmee N-beschermde amino-aldehyden bij kamertemperatuur racemiseren en de in het artikel aangeduide doelstelling om deze verbindingen optisch zuiver in handen te krijgen, is het als een ernstige tekortkoming te beschouwen, dat Freeman Stanfield et al. hieraan vrijwel geen aandacht schenken.

   Freeman Stanfield et al.(1981). J.Org.Chem.46,4797.

7. Het gevaar van optreden van recombinaties tijdens DNA cloneringsprocedures, vooral indien het te cloneren DNA repetitieve sequenties bevat, wordt gedemonstreerd door Shih & Weinberg, die na clonering eindigen met een 15 kbp EcoRI DNA fragment waarop behalve een onc gen ook Alu-repetitieve sequenties liggen, i.p.v. met het 25 kbp EcoRI DNA fragment zoals dat in het cellulaire DNA wordt aangetroffen.

   Shih,C. & Weinberg,R.A.(1982). Cell 29,161.

8. De bespreking van werken van Dostoyevsky door Nabokov voegt weinig toe aan de bestaande opvattingen. Integendeel, met zijn hoofdstelling omtrent het ontbreken van een karakter bij Dostoyevsky's hoofdpersonen gaat hij voorbij aan de ontwikkeling van hun pluriforme overtuiging naar een monistische wereldvisie tijdens de handelingen in de romans.

   Nabokov,V.(1981). Lectures on Russian Literature. Harcourt Brace Jovanovich, New York.

9. De opvatting omtrent het werk van auteurs, die door middel van errata wijzigingen introduceren in een eerder gepubliceerd artikel kort nadat over het onderwerp van dat artikel een belangrijk congres is geweest, wijzigt zich mede.

   Horn et al.(1981). Cell 25,37.
   en Cell 28,195(1982).

10. In zijn ijver het ongelijk van feministen te bewijzen, gaat 't Hart wel erg ver, als hij beweert dat vrouwen geen enkele rol speelden bij de totstandkoming van Beethoven's meesterwerken.

    't Hart,M.(1982). De vrouw bestaat niet. De Arbeiderspers,Amsterdam.
    "Beethoven".(1963). Verlag Kurt Desch GmbH, München.

11. Perler et al. en Efstratiatis et al. tonen aan dat de mate van silent site substitutie (1.5 %/miljoen jaar) na 100 miljoen jaar van divergentie afneemt tot deze gelijk wordt aan de mate van replacement site substitutie (0.1 %/miljoen jaar). Uit vergelijking van de nucleinezuursequenties van c-mos(muis) en c-mos(mens) gepubliceerd door Van Beveren et al. en Watson et al. blijkt dat voor c-mos de maten van silent site- en replacement site substitutie respectievelijk 0.12 en 0.16 %/miljoen jaar zijn. Dit suggereert dat een oer c-mos gen gezocht moet worden bij species die tenminste 100 miljoen jaar voor de divergentie muis/mens geleefd hebben.

    Perler et al.(1980). Cell 20,555.
    Efstratiatis et al.(1980). Cell 21,653.
    Van Beveren et al.(1981). Cell 27,97.
    Watson et al.(1982). Proc.Natl.Acad.Sci.USA 79,4078.

12. De beschrijving door Berlin van de periode 1848-1856 in Rusland kan - helaas - zonder wijzigingen overgenomen worden ter karakterisering van de grote problemen van de huidige Russische intelligentsia.

    Berlin,I.(1981). Russische Denkers. De Arbeiderspers, Amsterdam.

Nijmegen, 6-1-1983.